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A STUDY OF DOPAMINE ACETYL TRANSFERASE IN *DROSOPHILA MELANOGASTER.*

By

BRUNO J. M. MARANDA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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DEPARTEMENT OF GENETICS

EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "A study of Dopamine Acetyl Transferase in Drosophila melanogaster," submitted by Bruno J.M. Maranda in partial fulfilment of the requirements for the degree of Master of Science.

Supervisor: .....

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External Examiner: .....

Date October 10/75.....

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ABSTRACT

In this thesis, the biochemical and biological properties of dopamine acetyl transferase (DAT) in Drosophila melanogaster are described.

A sensitive spectrophotometric assay for the enzyme has been developed. The biochemical characterization of DAT has been initiated, including optimum pH and temperature, stability, Km, cofactor and ion requirements. A partially purified enzyme preparation has been obtained by ammonium sulfate fractionation and gel filtration on Sephadex G-200. The molecular weight of the partially purified enzyme was estimated to be 28,000 and confirmed by sucrose density gradient studies. In an attempt to eliminate the loss of enzyme activity which occurs during the purification procedure, affinity chromatography was developed as an alternative. DAT peak activity was found to occur at pupariation and eclosion; the enzyme is located, apparently, in the oenocytes. A genetic investigation of DAT, including activity of the enzyme in various mutants and an attempted localization of the structural gene for DAT, is also described.

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CHAPTER 1

INTRODUCTION

> "Differentiation is the outward and visible sign of selective gene activity, the reflection of a change in the cell's biochemical repertoire as a consequence of the release of information encoded in one dimensional sequences." (Allen,1965)

Correlations between the appearance of specific proteins and cellular differentiation have been reported in several instances ranging from bacterial sporulation (Brady,1973) and "stalk formation" in slime molds (Firtel,1973) to antibody formation (Hood,1970).

Since it is now accepted that the genetic information contained in the nucleus of a single cell from a complex organism is identical to the genetic information found in every somatic cell within the same organism (Gurdon,1962; McCarthy and Hoyer,1964) (although current studies on antibody formation, which indicate somatic mutation may be an important feature of generating diversity (Hood,1970), may affect the

generality of this statement somewhat), selective gene activity must be invoked to explain the facts that most of the genome in higher cell types seems inactive (Bonner, 1968) and that various RNA species are synthesized in different cell types (McCarthy and Hoyer, 1964).

Of the extracellular agents which trigger selective gene activation, hormones certainly seem to be among the most important. Discussions of hormone action usually focus on whether regulation is occurring at the transcriptional level in the nucleus or at the translational level in the cytoplasm. In prokaryotes, it has been proposed that regulation of gene function is dependent on the control of the mRNA specified by that gene (Jacob and Monod, 1961), and the utilization of lactose in E. Coli is the classic example of such a transcriptional control mechanism (Beckwith - The Lactose Operon, 1970)

The success of the Jacob and Monod model in explaining gene regulation in other prokaryotic systems, most notably the lambda bacteriophage (in "The Bacteriophage Lambda", A.D. Hershey ed. 1971), led many to postulate transcriptional controls were functioning in

higher organisms (Ohno, 1973). However, experiments with mammalian tissues exposed to hormones *in vitro* suggest that enzyme synthesis in higher organisms cannot be controlled exclusively at the transcriptional level (Tomkins et al., 1966). A model has been proposed by Tomkins (1969) in which hormones might regulate a repressor which both inhibits messenger translation and promotes messenger degradation. During the "inducible" phase of the cell cycle, the presence of the hormone (the "inducer"), causes the gene for tyrosine aminotransferase to be continuously transcribed into mRNA, which is then translated into the protein. A repressor is also produced by the regulatory gene but its action is inhibited by the inducer. When no inducer is present, it is postulated that the repressor combines with the messenger, preventing its translation into protein, and promoting the degradation of that same messenger. During the non-inducible phase of the cell cycle, the transcription of both the structural and regulatory gene is repressed independent of the inducer, but since the repressor is absent, the translation of the still present mRNA continues. Much evidence in support of this model has been presented (see Tomkins, 1972, for references) yet a controversy exists at present (Kenney et al., 1973). Without going into this , it is sufficient to say that

even in the case of inducible tyrosine aminotransferase, which was singled out since it is the best characterized eukaryotic system, the absence of genetic methodology is a major stumbling block to further progress on the mechanism of hormone action.

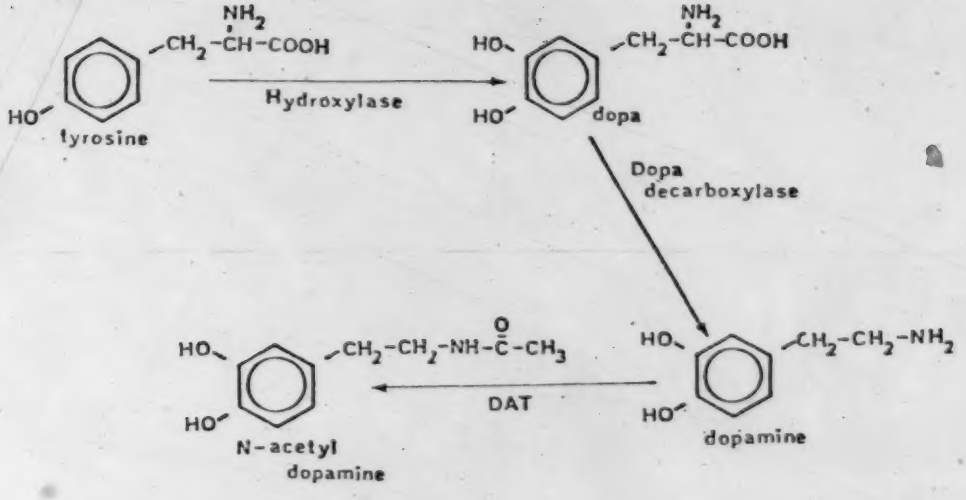
A few studies on hormone action in a genetically well defined organism have been reported (Fristrom et al., 1969; Chihara et al., 1972; Mandaron, 1970, 1971, 1972) but more complete investigations in an organism like Drosophila would be desirable.

In the insects, many of the morphological changes which occur during metamorphosis are under hormonal control (Doane, 1973; Wyatt, 1972). As demonstrated by various experimenters beginning with Fraenkel in 1935 and later by Karlson and Sekeris (1964), sclerotization (the hardening and darkening of the cuticle) of the posterior part of a Calliphora larva is prevented by ligation if performed prior to pupariation. Furthermore, sclerotization could be induced in the ligated portion following the injection of ecdysone, the molting hormone. However, ecdysone is not the only hormone that controls sclerotization. Bursicon has been proven to be the hormone responsible for cuticle maturation in newly

emerged blow flies and in various species of newly molted nymphs (Fraenkel and Hsiao, 1962, 1965; Cottrell, 1962). Furthermore, another hormone, a pupariation hormone, distinct from ecdysone and bursicon, that affects the tanning process, has been proposed (Zdarek and Fraenkel, 1969). Hence, it appears that sclerotization is not a simple process. However, as it has been relatively well defined biochemically (Karlson and Sekeris, 1964, 1966a ; Hopkins et al., 1971; Andersen, 1970; Bodnaryk, 1970, 1971), this system appears to be ideal for studying the action of hormones in Drosophila, and a simplified description of the biochemicals events responsible for sclerotization will be given.

Through oxidation, decarboxylation and acetylation, tyrosine is converted to N-acetyl dopamine in the third instar larva of Calliphora (fig. 1). With perhaps minor modifications, this pathway is the same in Drosophila melanogaster (Hodgetts and Konopka, 1973). N-acetyl dopamine, the precursor of o-quinone, the natural tanning agent, goes into the cuticle where it is oxidized (Karlson et al., 1962; Sekeris and Karlson, 1962,1964), and hardening is achieved by the interaction of cuticular proteins with tyrosine derived o-quinones. A network of proteins and chitin is produced by the crosslinking of

Fig. 1. The sclerotization pathway in Calliphora.



N-acetyl dopamine is transformed into benzo-quinones that are incorporated into the cuticle.

peptide chains, involving probably some chitin molecules (Brunet, 1965; Hackman and Goldberg, 1967). On the other hand, as demonstrated recently in our laboratory, beta-alanine also appears to be necessary in conjunction with o-quinones for normal (wild type appearance) tanning in Drosophila melanogaster (Hodgetts and Choi, 1974). In adults, beta-alanine is postulated to interact with indole quinones resulting from oxidation of dopamine present prior to eclosion, and thereby prevent the melanization of those quinone molecules. Beta-alanine being absent in the mutant black causes a dark phenotype due to excessive melanization.

The question has been asked whether hardening and darkening of the cuticle are two independent processes or whether they are the results of the same chemical action. Evidence has been given that two mechanisms, one giving a colorless cuticle and the other a dark brown cuticle, exist in insect cuticle, and that both mechanisms can be active simultaneously and can utilize the same substrate, N-acetyl dopamine (Andersen and Barrett, 1971). Two different enzymes appear to be responsible , one for the formation of the colorless cuticle and the other for the dark cuticle. However, the possibility that both enzyme activities reside in a same and unique enzyme cannot be

disregarded and this has not been resolved yet (Andersen, 1974).

The enzymes in the pathway leading to N-acetyl dopamine from tyrosine increase in activity during the period corresponding to or just preceeding puparium formation. Furthermore, dopa decarboxylase activity has been correlated to the increased titer of ecdysone, characteristic of this period (Shaaya and Sekeris, 1965; Karlson and Ammon, 1963) and a direct proof that ecdysone is responsible for the appearance of dopa decarboxylase has been obtained by injection of ecdysone into ligated animals and by in vitro experiments (Chen and Hodgetts, 1974). Since inhibitors of protein and RNA synthesis prevented the appearance of dopa decarboxylase activity in these studies, it appears that both protein and RNA synthesis are important aspects of the hormonal induction of the enzyme.

Because of the interest in the regulation of dopa decarboxylase, we set out in this study to gain information on DAT (dopamine acetyl transferase), the enzyme which follows dopa decarboxylase in the sclerotization pathway. This enzyme has not been studied in detail in any insect and we felt a comparison of its

properties to those of dopa decarboxylase would be useful. We choose Drosophila melanogaster for these studies since our long term goal is to elucidate the genetic mechanisms which underlie the regulation of the activities of enzymes.

In this thesis, we shall describe in detail a new assay for the enzyme dopamine acetyl transferase and certain of its biological and biochemical properties, which provides a basis for possible future studies on the effects of hormones on the activity of the enzyme.

CHAPTER 2

Materials and Methods.

1) Maintenance of Stocks.

The mutants *black* and *ebony* and the Canton-S wild type strains of *Drosophila melanogaster* came originally from the Pasadena collection. The stocks were maintained on a yeast-sucrose medium (Nash and Bell, 1968) and kept at 25°C in constant darkness.

2) Preparation of Extracts.

Routinely, crude extracts of the enzyme were prepared by homogenizing 200mg (live weight) of larvae or adults in 0.05M Tris-HCl buffer, pH7.4 at 22°C, which contained .001 M phenyl thio urea (PTU) to inhibit phenol oxidase activity (Mitchell, 1964). The larvae were separated from the food by floatation on 5% sucrose. The grinding was performed in a small glass conical grinder (Bellco) in volumes varying from 0.2 to 1.0 ml until no solid material was observed. The homogenate was then centrifuged at 7700 x G for 10 minutes, and the supernatant was recovered and kept on ice for assaying.

On occasion, extracts were prepared which unexpectedly contained no activity. While we have no explanation for this result, it appears that the duration and method of grinding are critical.The duration of grinding should be kept as short as possible. As will be argued later, most of the enzyme activity appears to be located in the oenocytes, large cells located just beneath the epidermal cells. Apparently, very slight grinding is sufficient to break these cells loose and to liberate the enzyme. For extracts containing less than 20 organisms, several twists with the grinder handle are sufficient. Moreover, it seems rather important to avoid an introduction of air bubbles and to prevent foaming while grinding. To minimize surface denaturation which we consider to be the most likely explanation for the loss of activity, glycerol at a concentration of 10% and bovine serum albumin at a concentration of 1 mg/ml were added to the extract buffer in most of the later experiments reported here.

The grinding technique and duration seem to be more critical for larvae than for adult flies. No difference in activity was observed in extracts of young adults ground for 15 seconds or 2 minutes while a 2 minutes

grinding time often decreased the activity in extracts of third instar larvae (Table 1).

A particularly efficient way of grinding large quantities of flies consisted of freezing the flies in liquid nitrogen and grinding them in liquid nitrogen to a fine powder in a precooled mortar. A more convenient way (and nearly as efficient) though, was to make a slurry containing 2.0 gm of flies in 10.0 ml of Tris-HCl buffer and to grind them in a large mortar with a pestle.

3) Enzyme Assay.

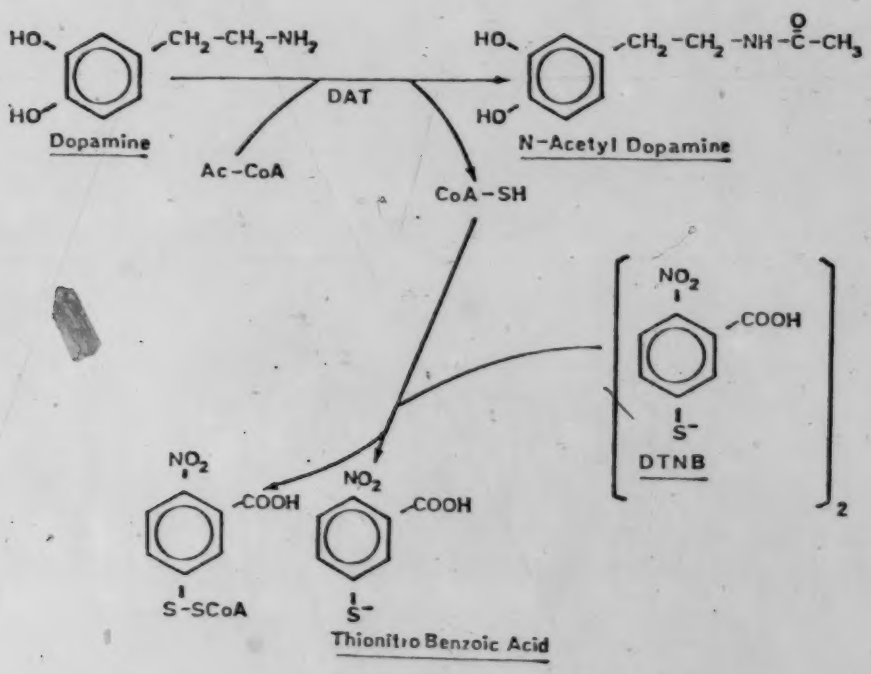
The basis for the assay is a spectrophotometric monitoring of the appearance of thionitrobenzoic acid which has an absorbtion maximum at 412 nm. The method is based on a disulfide interchange between the CoA liberated by DAT from acetyl CoA and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1958, 1959 and Alpers et al.1965) (fig. 2). The acetyl transferase assay was carried out, at room temperature, in micro cells (minimum volume 0.20 ml) of 10 mm light path in a double beam Beckman DB spectrophotometer, which allows to continuously correct for the absorbance changes in the blank..

TABLE 1. Effect of grinding time on the activity of DAT in crude extracts.

Duration of grinding	DAT activity (Units/min/mg wet weight)	
	White puparium stage	Young adults
15 sec	0.0202	0.0193
30 sec	0.0196	0.0207
1 min	0.0175	0.0191
2 min	0.0149	0.0217

White puparia and 0-3 day old adults were ground in Tris-HCl buffer 0.05M, pH 7.2, containing 1 mM PTU, at a concentration of 200 mg/ml. The grinding was performed in small conical glass grinder (Bellco) at 4° C. Assay conditions for the determination of the enzyme activity were as described in Materials and Methods.

Fig. 2. Dopamine acetyl transferase (DAT) assay.



The activity of DAT is measured by the appearance of thionitrobenzoic acid which has an absorption maximum at 412 nm.

At the outset, the following assay mixture was used based on preliminary studies by Hodgetts and Konopka (1973):

-50 ul tris-HCl buffer 0.05 M, pH 7.4 at room temperature

-25 ul PTU (1.78 mg/ml, ddw) at room temperature

-50 ul Acetyl CoA (0.44 mg/ml, ddw) at room temperature

-50 ul crude extract in Tris-HCl 0.05 M pH 7.4 at 4°C

-25 ul dopamine (7.5 mg/ml, ddw) at 4°C

-50 ul DTNB (5,5' dithiobis(2-nitrobenzoic acid)) (0.94 mg/ml Tris-HCl buffer) at room temperature

The O.D. 412nm of the sample was measured against a blank in which double distilled water (ddw) was substituted for the dopamine solution. The reagents were added to the cuvette in the order presented here.

Several slight modifications of the above procedure were made. Studies on the kinetics of the enzyme (see chapter 3) revealed that the acetyl CoA concentration in the above reaction mixture was about 2 times the Km and

therefore adequate. However, the concentration of dopamine was lowered to 0.75 mg/ml since this corresponds to twice the Km value for dopamine. It should be pointed out that the first assays were performed using an aqueous solution of dopamine. However, since it was found that this solution spontaneously melanized upon standing, 0.001 N HCl was substituted for water as the solvent. Even after several hours standing at room temperature, an acidic dopamine solution showed no signs of oxidation. Therefore, the substrate dopamine was routinely prepared in a 0.001 N HCl solution , and 0.001 N HCl was used in the blank. Finally, studies on the optimum pH of the enzyme (chapter 3) indicated a pH value of 7.2 was more appropriate.

Using these assay conditions which are nearly optimal (see chapter 3) and which have been used as the standard assay procedure, enzyme activity was linear for at least 10 minutes (fig. 3) and proportional to the concentration of extract (fig. 4). Even at the high concentrations of enzyme which were encountered in partially purified extracts, the reaction rate was proportional to the enzyme concentration up to an activity which produced an optical density change of 0.5 units/min. With such concentrations, the

Fig. 3. A typical chart recording from the spectrophotometric assay of DAT activity.

The activity was routinely calculated from the third to the eighth minute which corresponds generally to the linear section of the recording.

Assay conditions are similar to those described in Materials and Methods.

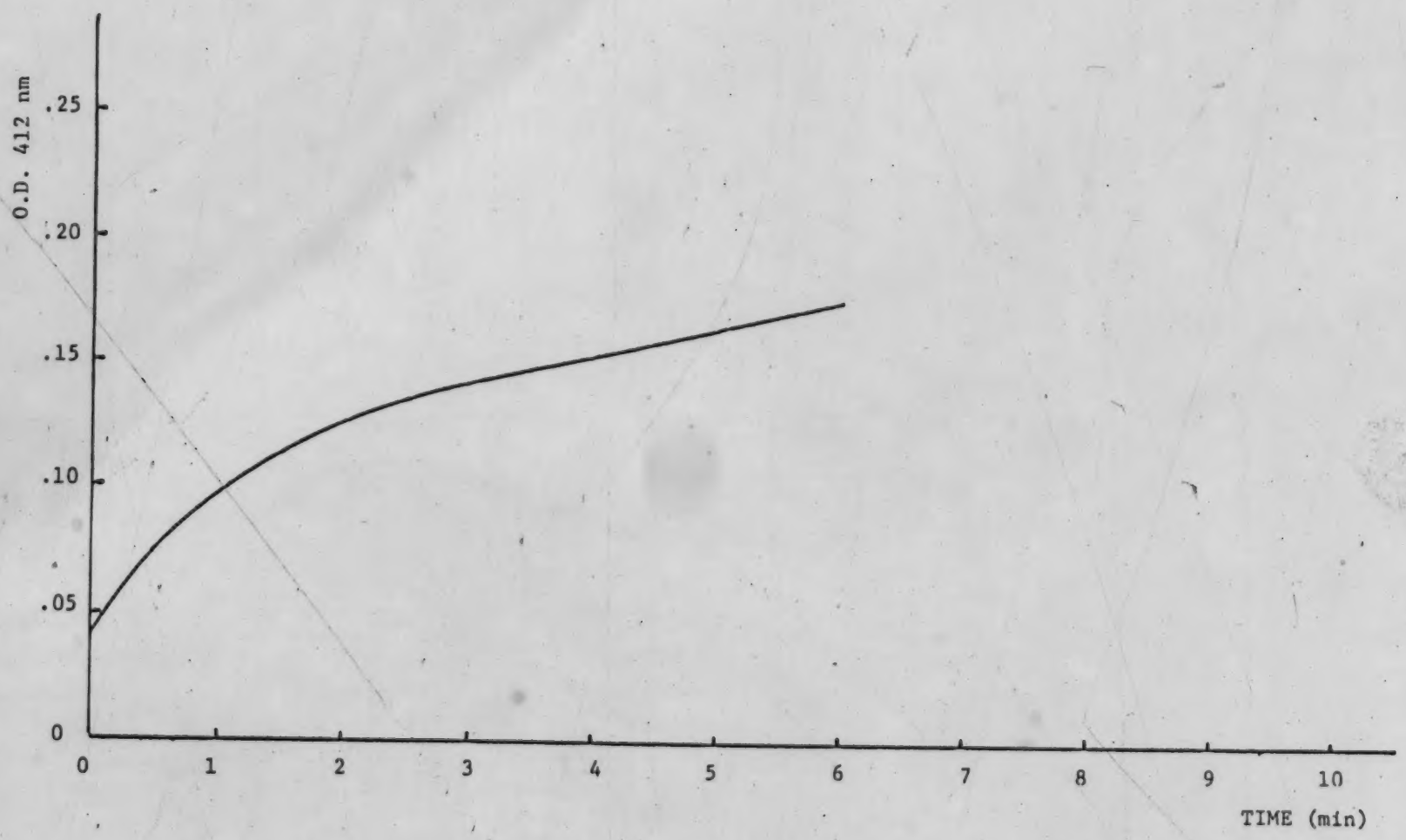
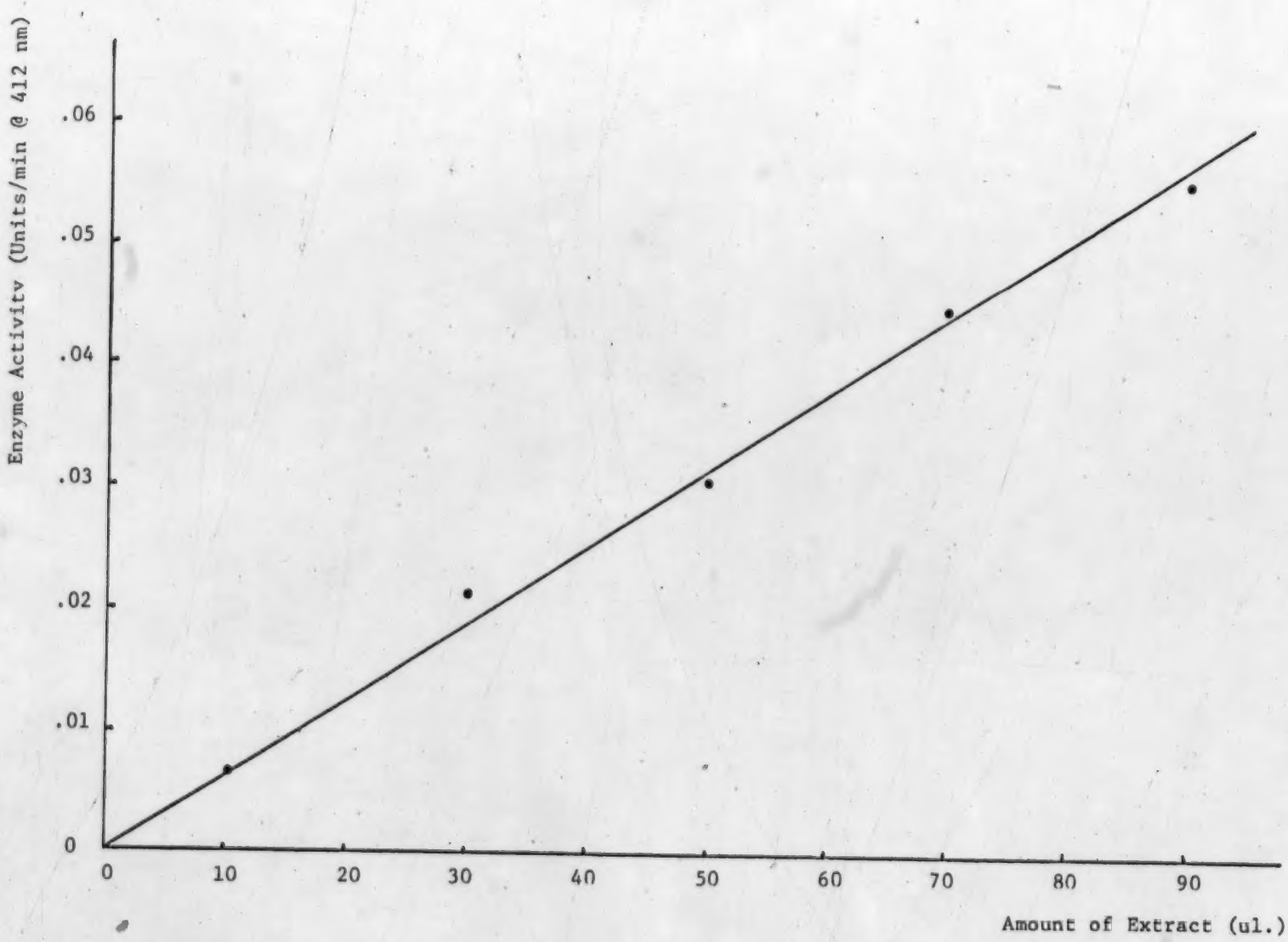

O.D. 412 nm
.25
.20
.15
.10
.05
0
0
1
2
3
4
5
6
7
8
9
10
TIME (min)

Fig. 4. Enzyme activity vs enzyme concentration.

A crude extract was prepared by grinding 0-3 day old adult flies in a proportion of 200 mg flies per milliliter of grinding buffer (Tris-HCl, pH 7.2, 0.05 M containing 0.1 M PTU).

Assay conditions for the determination of the enzyme activity were as described in Materials and Methods.


Enzyme Activitv (Units/min @ 412 nm)
.06
.05
.04
.03
.02
.01
0
0
10
20
30
40
50
60
70
80
90
Amount of Extract (ul.)

spectrophotometric determination was stopped after 2 minutes or when O.D. Reading of 1.0 was reached. In any cases, the reactions were rarely run for more than 10 minutes and the linear portion of the slope, usually from the third to the eighth minute, was used to determine the enzyme activity. The non-linear portion of the slope, at the beginning of the reaction, varied in length and aspect. We have no explanation for the inconsistencies characteristic of the early stage of the spectrophotometric determination.

4) Sephadex column chromatography.

G-25, G-150 and G-200 Sephadex gels (Pharmacia Fine Chemicals,Montreal,Quebec) were prepared as follows. Five gm of G-25 were soaked in 300 ml of 0.05 M Tris-HCl buffer (pH 7.2) for 3-5 hours at room temperature. For G-150 and G-200, 20 to 25 gm of the dry gel were soaked in a liter of Tris-HCl buffer, for 3 days at room temperature or for 5 hours at 100°C. Once the gels were fully hydrated, the fine particles were removed by successively washing the gel with Tris-HCl buffer. Air bubbles trapped in the gel slurry during preparation were removed under vacuum with gentle agitation. For dialysis purposes, G-25 was packed into a small column (0.9 x 10

cm) and equilibrated with 0.05 M Tris-HCl pH 7.2 at 4°C. (with or without PTU). The same column was used until major contamination by debris was observed.

For gel filtration column chromatography and molecular weight determinations, the excess buffer used to swell the G-150 or G-200 gels was removed until a thick slurry remained. The slurry was then carefully poured into a slanted column (to avoid trapping air bubbles). Different sizes of column were used and varied from 1.2 x 55 cm to 1.2 x 100 cm. The columns were connected to a buffer reservoir consisting of a Mariotte flask and the gel was packed under an operating pressure not exceeding 12 cm. The column was further equilibrated by the passage of at least 10 times the dead volume of 0.05 M Tris-HCl buffer.

The void volume of the column was determined with 0.2% blue dextran-2000 (Pharmacia Fine Chemicals). In order to obtain good separation of the proteins, the amount of sample loaded on the column should be less than 2% of the total bed volume (Andrews,1964). Therefore, samples were always applied in volumes less than 1.0 ml. Following sample application, the column was eluted under an operating pressure of 12 cm with a volume of buffer exceeding twice the bed volume of the column. For

molecular weight determinations, the flow through the column was regulated by the use of a peristaltic pump operating at a rate of 10 ml/hour. The volume of fractions collected was under most circumstances 0.9 ml. The columns were successfully reused if extensively washed with Tris-HCl buffer after each run.

5) DEAE column chromatography

DEAE (diethyl amino ethyl cellulose - W. and R. Balston, England) was prepared by mixing 50 gm of DEAE with 600 ml of 0.5 M NaOH for 1 hour at room temperature. NaOH was removed by suction on a Buchner funnel and the pH of the suspension was reduced to 8.0 by repeated rinses with double distilled water. The DEAE was resuspended in 600 ml of 0.5 M HCl, stirred at room temperature and washed with double distilled water to pH 4.0 . Then, the DEAE was resuspended in 300 ml of 0.05 M Tris-HCl buffer (pH 7.2) , stirred for 30 minutes and titrated with 6 N KOH until a stable pH of 7.2 was obtained. A 1.2 x 15 cm column was packed with the treated DEAE and equilibrated by passing through the column 300 ml of 0.05 M Tris-HCl buffer, pH 7.2, at 4°C, under an operating pressure of 10 cm.

6) Estimation of protein concentration.

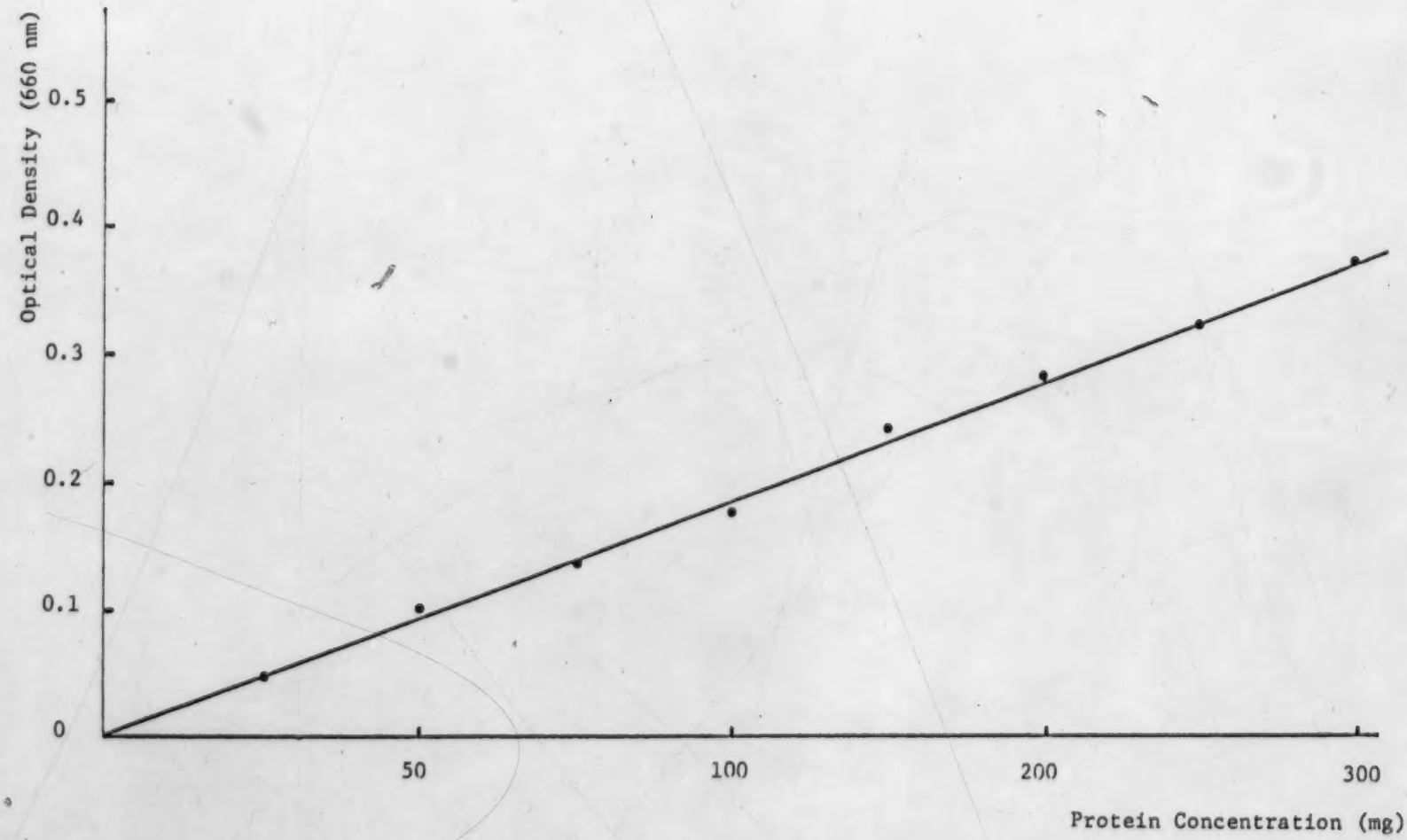
The concentration of protein in each extract was determined by the method described by Lowry et al. (1951), although, preliminary estimates were sometimes obtained by readings of the optical density at 280 nm. Several difficulties were encountered in applying the Lowry method and these have been discussed by Chen (Ph.D. Thesis, 1973). Briefly, both PTU and Tris react with the Folin reagent to give false Lowry positive reactions. This is very serious in the case of PTU at concentrations in the vicinity of .001 M. The slight false Lowry positive reaction observed when the Folin reagent was added to Tris-HCl Buffer was accounted for by substracting appropriate blank values obtained by substituting the Tris-HCl buffer for the sample. The intensity of the blue color which develops in the procedure is a function of time. Therefore, throughout all the assays, an incubation time of one hour was used. All the reagents except the Folin reagent were renewed every month and after renewal, a standard protein concentration curve was prepared using bovine serum albumin. A typical standard curve is shown in figure 5.

7) Sucrose density gradient centrifugation

Fig. 5. Typical standard curve for the estimation of protein concentration by the Lowry method.

Standard curves were prepared according to the method of Lowry et al. (1951) using a solution of bovine serum albumin (BSA) in the sample buffer (0.05 M, pH 7.2, Tris-HCl).

The O.D. 660 nm was measured 60 minutes after the addition of the Folin reagent.


Optical Density (660 nm)
0.5
0.4
0.3
0.2
0.1
0
50
100
200
300
Protein Concentration (mg)

Density gradient centrifugation was carried out on the partly purified fraction following ammonium sulfate treatment. The appropriate ammonium sulfate cut was redissolved in Tris-HCl buffer, pH 7.2, 0.05 M with no PTU and dialysed by passing through a small Sephadex G-25 column. A sample (0.2 ml) of the dialysed material was layered on a 4.6 ml continuous gradient of sucrose (5 to 20%), in Tris-HCl buffer, pH 7.2, .05M, and centrifuged at 40,000 rpm in a Spinco SW50.1 rotor for 18 hours at 4°C. The fractions were collected by puncturing the bottom of the tube and counting the number of drops, the speed of delivery being controlled by air injection on the top of the tube. The location of DAT was determined with standard assay procedures.

8) Affinity chromatography

I- Glass beads

A) Preparation of aryl amine glass beads (fig. 6)

The procedure of H.W. Weetall (1969,1971) was followed with slight modifications. Arylamination was performed on GAO-3940 porous glass particles with an average pore diameter of 550 Angstroms, obtained from Corning Biological Products (fig. 7). To one gram of the GAO-3940

Fig. 6. Procedure for the attachment of dopamine to glass beads.

GAO-3940 glass beads (Corning) with a pore diameter of 550 Angstroms are treated with p-nitrobenzoyl chloride and triethylamine (1). The nitrated glass beads are reduced (2) with sodium dithionite, and the arylamine beads diazotized (3) in the presence of HCl with sodium nitrite. The diazotized beads are coupled with the aromatic amine (4) to produce glass beads with dopamine attached. Details of the procedure are given in the text.

Fig. 6.

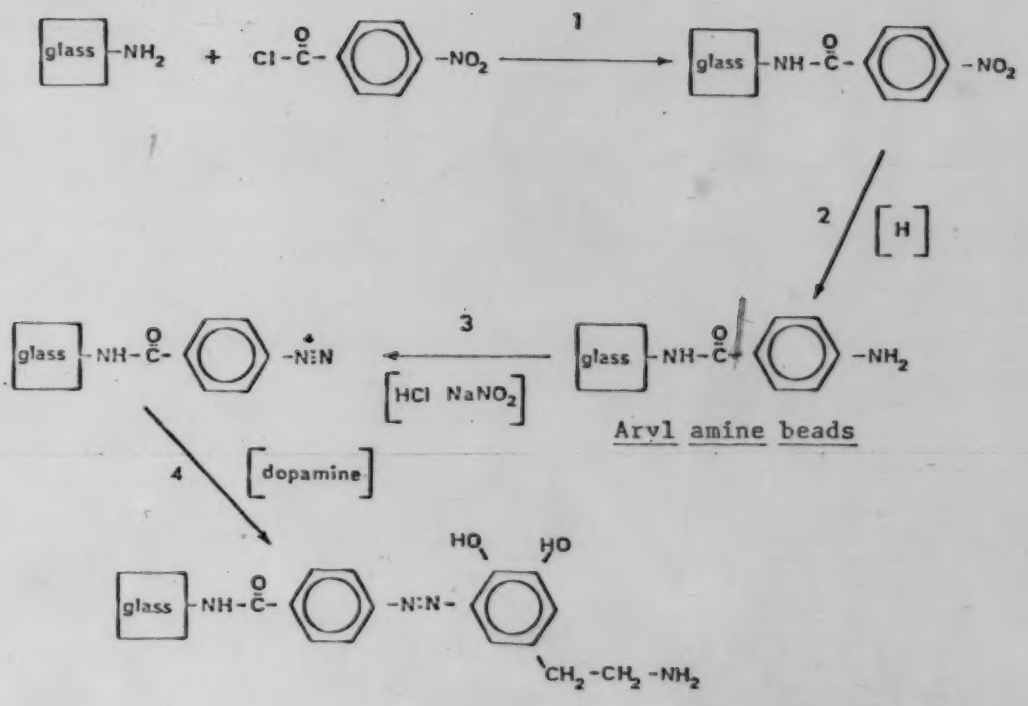
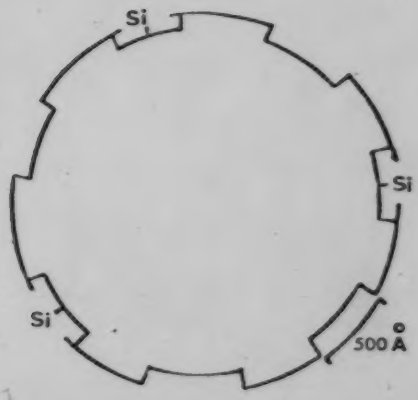

glass
-NH2
+
Cl-C-
O
-NO2
1
glass
-NH-C-
O
-NO2
2
[H]
glass
-NH-C-
O
-NH2
Aryl amine beads
3
[HCl NaNO2]
glass
-NH-C-
O
-N:N
4
[dopamine]
HO
HO
glass
-NH-C-
O
-N:N-
CH2-CH2-NH2

Fig. 7. Porous glass beads.



Schematic representation of the physical structure of the GAO-3940 porous glass beads as received from Corning Glass Works.

A proposed structure of dopamine linked to glass beads is illustrated in figure 15a.

glass beads, 10 ml of a chloroform solution containing 100 mg of p-nitrobenzoyl chloride (previously recrystallized with carbon tetrachloride) and 50 mg of triethylamine were added. The reaction mixture was refluxed for one hour with "boil easy" porous particles. The solution was then decanted and the beads washed three times with chloroform. The chloroform was then removed by air drying. The nitrated glass beads were reduced by adding 10 ml of a 10% aqueous sodium dithionite solution and refluxing for 60 minutes. The reaction solution was decanted and the aryl amine glass beads washed three times with water.

B) Diazotation and azo coupling of dopamine (fig.6)
Based on the J.C. Venter procedures (1972,1973), the still moist aryl amine glass beads (approximately one gram) were placed in 20 ml 2 N HCl containing 0.250 gm of sodium nitrite. The preparation was allowed to stand at 0°C under vacuum for 30 minutes. The activated glass beads were then filtered and washed with 500 ml of ice cold double distilled water and placed immediately in 10 ml of 0.05 M Tris-HCl buffer, pH 7.2, containing 10 mg of non-radioactive dopamine and 10 ul of ^{14}C-dopamine (100 uCi/ml) (fig. 6).
The reaction mixture was allowed to stand for one hour at

25°C in the dark. The brown colored glass beads were then washed with 1.2 liters of 0.1N HCl. The washing solution was kept for assays on radioactivity and the glass beads were stored moist at 4°C. Before using, the treated beads were washed with Tris-HCl buffer, pH 7.2, 0.05 M to removed any detached dopamine.

II- Sepharose 4b beads (fig. 8)

The procedure of attaching dopamine to Sepharose 4b beads was a modification of that of Cuatrecasas (1970).

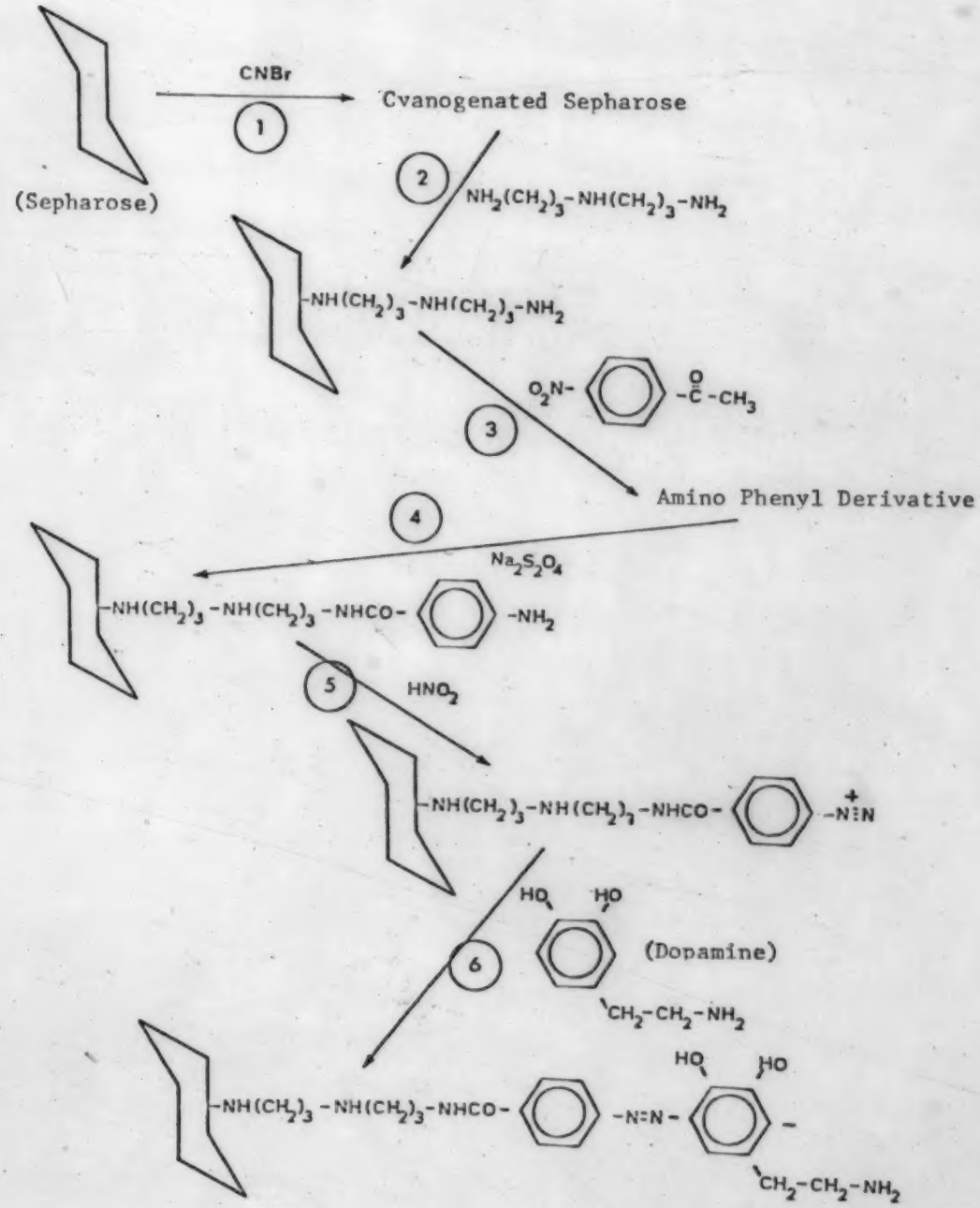
A) Preparation of amino ethyl Sepharose.

In a well ventilated hood, 250 mg of cyanogen bromide per milliter of packed gel was added rapidly To a Sepharose 4b suspension (Pharmacia Fine Chemicals). The pH of the reaction mixture was immediately raised to and maintained at 11.0 with NaOH (2.0 M). The temperature was maintained at approximately 20°C by adding pieces of ice as needed. The reaction was completed in 8 to 12 minutes as indicated by the cessation of proton release. A large amount of ice was then rapidly added to the suspension, which was transferred quickly to a Buchner funnel (coarse disc) and washed under suction with cold saturated borate buffer (pH 10.0), the volume of wash being 10 to 15 times that of the packed Sepharose. The washed, activated

Fig. 8. Procedure for the attachment of dopamine to Sepharose beads.

Diazonium-Sepharose derivatives are prepared by treating Sepharose-4B with cyanogen bromide (1) followed by 3,3'-diaminodipropylamine (2). This amino Sepharose is then treated with p-nitrophenylazide (3). The aminophenyl derivative is reduced with sodium dithionite (4) and then diazotized with nitrous acid (5). Ligands having phenolic or imidazole groups, like dopamine, react rapidly with diazonium Sepharose (6).

Fig. 8.


CNBr
1
Cyanogenated Sepharose
(Sepharose)
2
$NH_2(CH_2)_3-NH(CH_2)_3-NH_2$
$-NH(CH_2)_3-NH(CH_2)_3-NH_2$
O_2N-
$-\overset{O}{\overset{\|}{C}}-CH_3$
3
Amino Phenyl Derivative
4
$Na_2S_2O_4$
$-NH(CH_2)_3-NH(CH_2)_3-NHCO-$
$-NH_2$
5
HNO_2
$-NH(CH_2)_3-NH(CH_2)_3-NHCO-$
$-N\equiv N$
HO
HO
(Dopamine)
6
$CH_2-CH_2-NH_2$
$-NH(CH_2)_3-NH(CH_2)_3-NHCO-$
$-N=N-$
$CH_2-CH_2-NH_2$

Sepharose was then added to an equal volume of cold distilled water containing 2 mmoles of 3,3'diaminodipropylamine for each milliliter of Sepharose, previously titrated to pH 10 with 6N HCl. The entire procedure of washing, adding the ligand solution and mixing consumed less than 90 seconds , since the activated Sepharose is unstable. After reaction for 16 hours (the first 3-4 hours with gentle mixing) at 4°C, the gel was washed with large volume of double distilled water . This treatment results in a derivative having about 12 umoles of aminoethyl groups per milliliter of Sepharose.

B) Preparation of p-aminobenzamido ethyl Sepharose and attachment of dopamine via azo linkage.

3,3' diaminodipropylamine Sepharose beads in 0.2 M sodium borate buffer, pH 9.3 and 40% dimethyl formamide (v/v) are treated for one hour at room temperature with 0.07 M p-nitrobenzoyl azide. The substitution is completed as judged by the loss of color reaction with sodium trinitrobenzene sulfonate (Cuatrecasas, 1970). The p-nitrobenzamido ethyl Sepharose was then washed extensively with 50% dimethyl formamide and reduced by reaction for 40 minutes at 40°C with 0.1 M sodium dithionite in 0.5 M $NaHCO_3$, pH 8.5 . The washed p-

aminobenzamido ethyl Sepharose derivative, in 0.5 N HCl, can be diazotized by treating for 7 minutes at 4°C with sodium nitrite (0.1 M). To this diazonium-Sepharose derivative was added the non-radioactive dopamine (1 mg per ml of Sepharose) containing ^{14}C-dopamine (1 uCi per mg of non-radioactive dopamine) in solution in saturated sodium borate buffer, pH 10.0. The pH of the reaction mixture was adjusted to 10 with NaOH and allowed to react for 8 hours at 4°C. The treated beads were extensively washed with double distilled water and kept in suspension with Tris-HCl buffer 0.05 M, pH 7.2 . As before, the beads were washed once prior to using.

CHAPTER 3

Biochemical Characterization

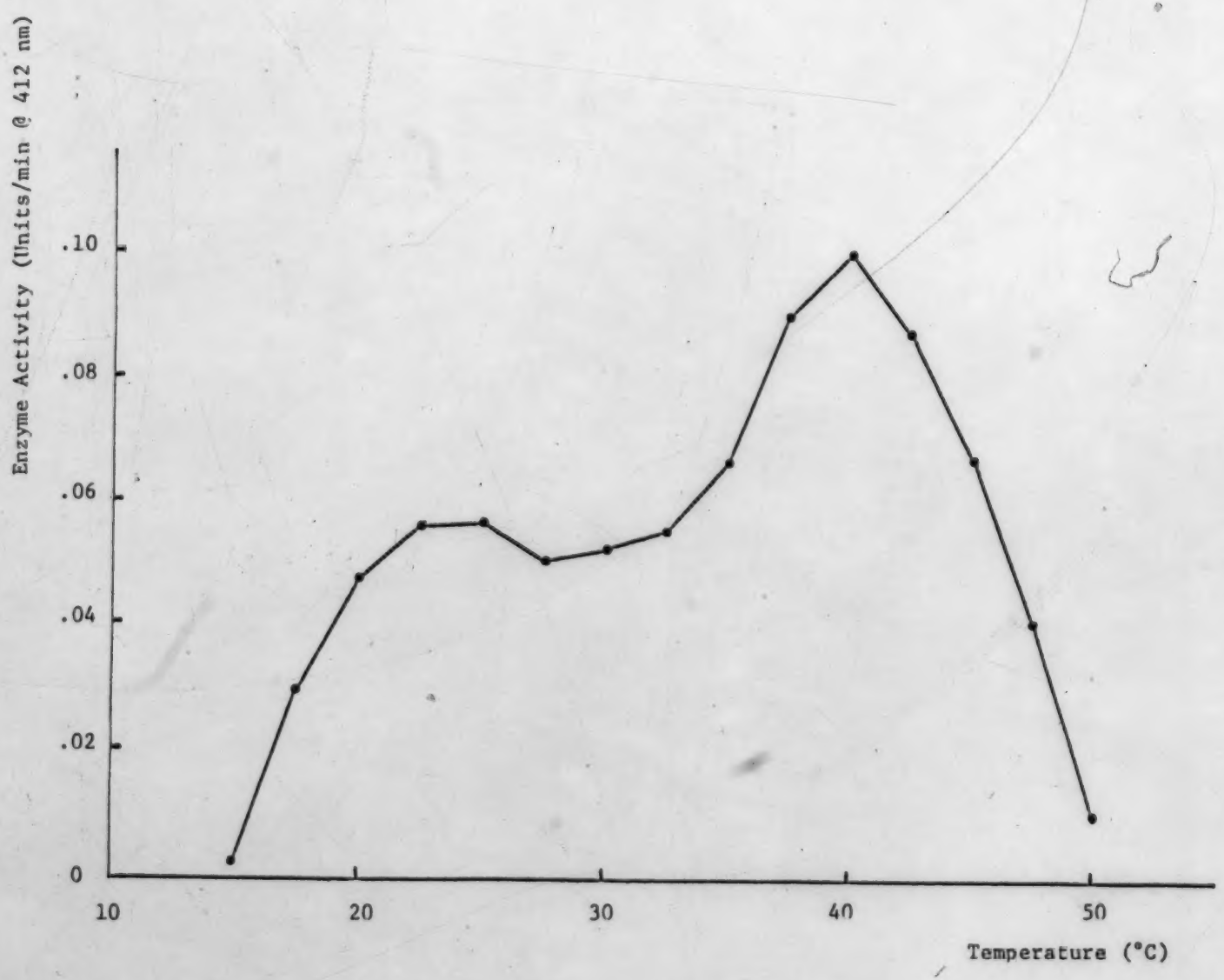
In the previous studies on DAT (see Karlson and Ammon, 1963; Hodgetts,R.B., unpublished), no attempt to investigate optimal substrate concentrations, cofactor requirements, temperature and pH optima was made. These were part of the subject of investigation in this thesis.

A) Optimum Temperature.

As illustrated in figure 9, the optimal assay temperature corresponds to 37-40°C. However, as no spectrophotometer with a temperature controlled chamber was available, all standard assays were subsequently performed at room temperature. The advantage of using an assay temperature of about 22°C is that enzyme inactivation, which becomes appreciable above 30°C (see following) does not occur. This bimodal curve is rather surprizing. This could be explained by an unknown artifact in the procedure, by the presence of two temperature optima or by the existence of two enzymes acting similarly on dopamine, but at different temperatures. These hypotheses have not been verified, however.

Fig. 9. Enzyme activity vs temperature.

This study was done with a crude extract of 0-3 day old adults, using standard assay conditions (see Materials and Methods). All reagents and the cuvettes were kept at the indicated temperature prior to assaying, since no spectrophotometer with a temperature-controlled chamber was available. The activity was then measured immediately after the addition of the final reagent.


Enzyme Activity (Units/min @ 412 nm)
.10
.08
.06
.04
.02
0
10
20
30
40
50
Temperature (°C)

B) Inactivation Temperature.

As shown in figure 10, the inactivation temperature is not unusual for a protein (Bernhard,1968), showing a rapid decrease in activity over 30°C and complete denaturation by 50°C. As a result, assay conditions resulting in temperatures higher than 30°C are to be avoided.

It should be pointed out that although the temperature of the cuvette increased noticably during an assay, the enzyme reaction was linear for at least 10 minutes (fig.3). This may of course be a reflection of an equilibrium between the rate at which the enzyme is denaturing and the increase rate of reaction.

C) Stability.

Both dialysed and non dialysed crude extracts of the enzyme were found to be stable for a relatively long period of time if kept on ice (fig 11). However, the activity of a non dialysed crude extract decreases with the number of times it is frozen and thawed, while dialysed extracts are quite stable . The addition of NaCl (0.1 M) to a crude extract tends to stabilize the enzyme during freezing and thawing, although NaCl has no effect on the enzyme activity in freshly prepared crude extracts. PTU (about 1 mM) seems to have the same

Fig. 10. Heat inactivation of DAT.

Samples from a crude extract of 0-3 day old adults were incubated at the appropriate temperature for 20 minutes prior to assaying.

Each sample was then brought back to 4° C and the assays were performed at room temperature using the standard procedure.

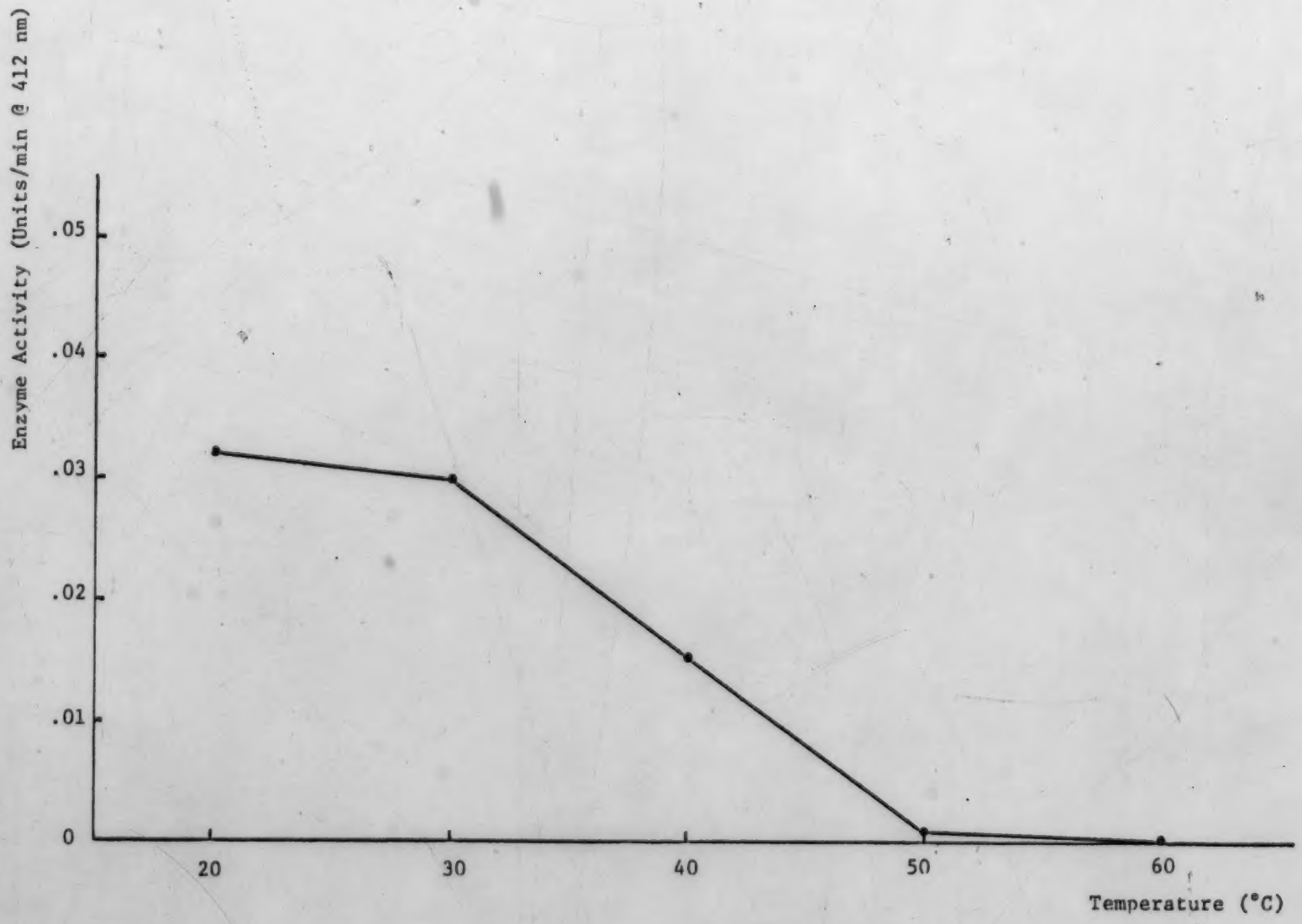
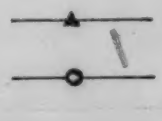

Enzyme Activity (Units/min @ 412 nm)
.05
.04
.03
.02
.01
0
20
30
40
50
60
Temperature (°C)

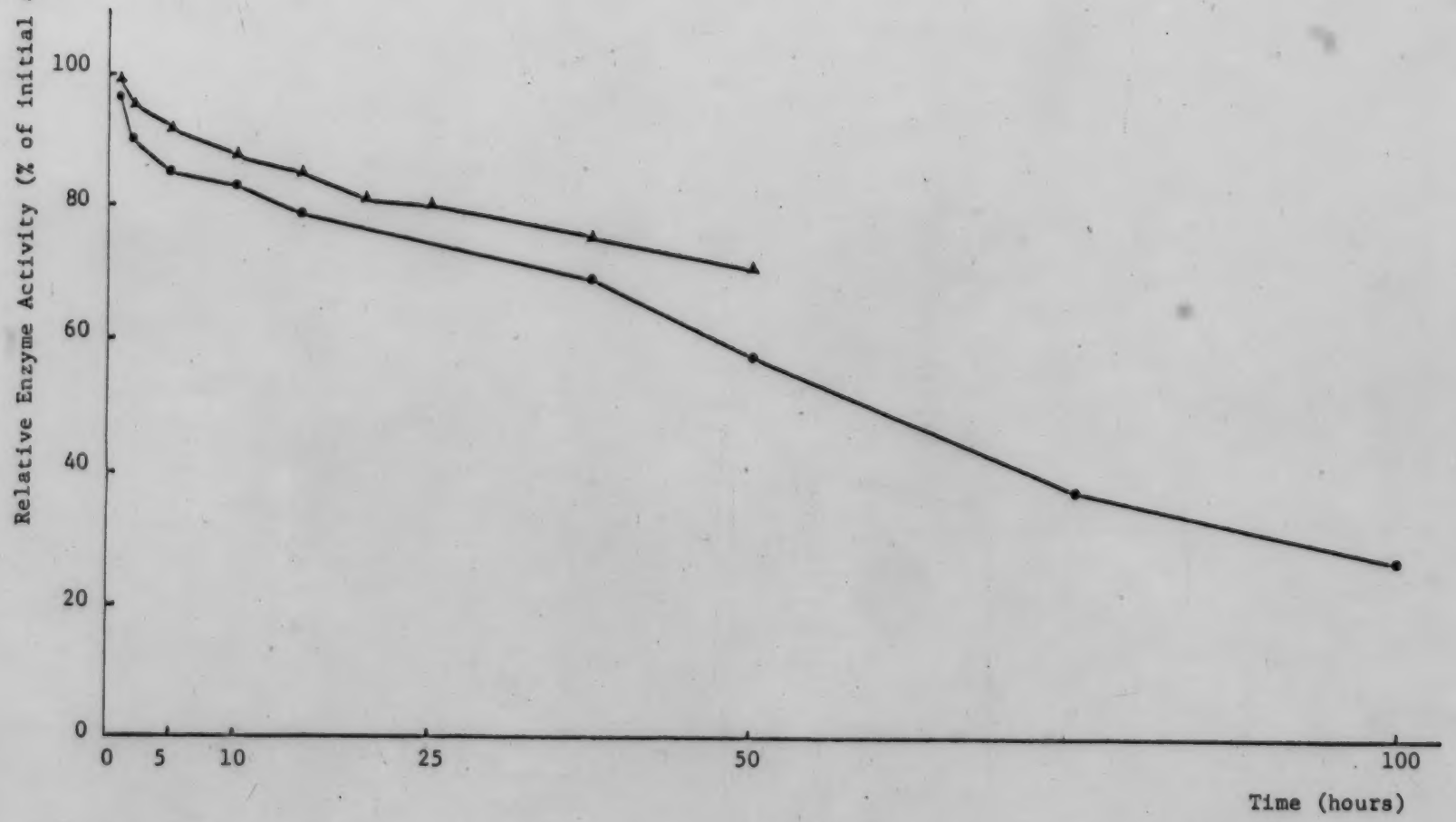
Fig. 11. Inactivation of DAT in crude extracts at 0° C.

A crude extract was prepared from 0-3 day old adults and part of this was dialyzed by passage over G-25 (see Materials and Methods). These two extracts were then assayed under standard conditions after standing for various times in an ice bath.



Dialyzed extract

Non-dialyzed extract


Relative Enzyme Activity (% of initial activity)
100
80
60
40
20
0
0
5
10
25
50
100
Time (hours)

stabilizing effect as NaCl.

The enzyme may be stored for long periods at -40°C, and in one experiment 62% of the original activity remained after more than 6 months.

D) Optimum pH.

The results of a study to determine the optimum pH of the enzyme are shown in figure 12. It should be pointed out that all the reagents were in solution in ddw (double distilled water) while the extract was made in a saline solution (0.1 M) containing PTU (1 mM). At low pH, the ionization of the thionitrobenzoic acid formed during the spectrophotometric assay decreases (pK=5.1; Ellman, 1958) and accordingly a decrease in the absorbance at 412 nm occurs. However, no attempt to correct the values of the enzyme activity at low pH was made. Because of the relatively low pK for the thionitrobenzoic acid, in the physiological range of pH, the errors which might be ascribed to variations in the degree of ionization will be negligible.

E) Cofactor Requirements.

None of the usual cofactors or metallic ions was found to enhance the enzyme activity (table 2).

Fig. 12. Optimum pH of the DAT.

For optimum pH determination, assay conditions were altered so that all the reagents were in double distilled water, the crude extract in saline solution (0.1 M) containing 1 mM PTU and buffers were of the appropriate pH value.

Tris-Maleate and Tris-HCl buffers were used to cover the entire range of pH extending from 2.0 to 10.0. Only the portion corresponding to maximum activity is shown.

0-3 day old adults were used and assays were performed at room temperature.

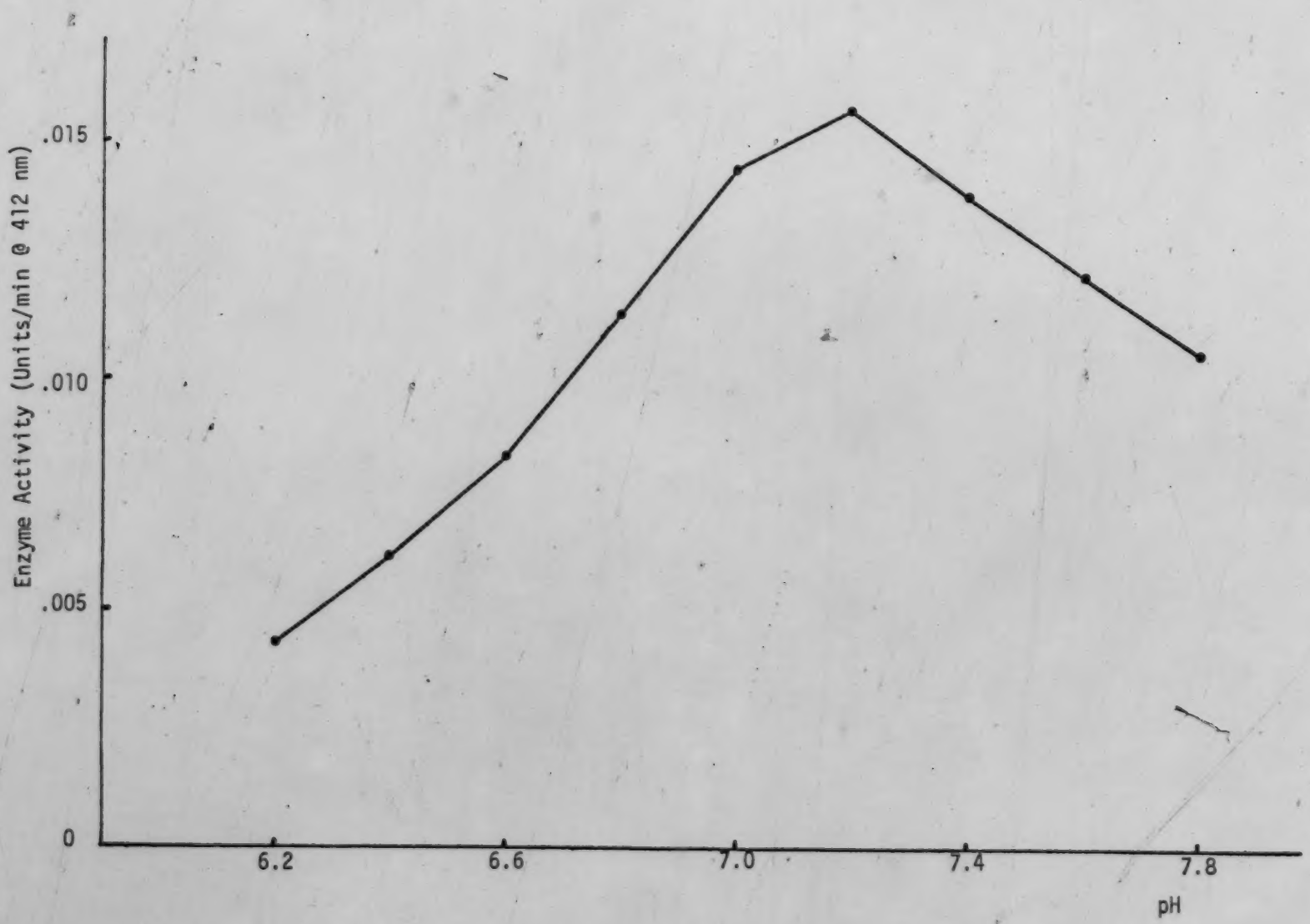

Enzyme Activity (Units/min @ 412 nm)
.015
.010
.005
0
6.2
6.6
7.0
7.4
7.8
pH

TABLE 2. Co-factor requirements.

A) Metallic ions	Concentrations	DAT activity (Units/min/mg)
Fe+++ (Cl---)	$1/10^3$ M	Fe+++ reacts with
	$5/10^3$ M	DTNB to form a
	$1/10^2$ M	yellow complex.
Mg++ (Cl--)	$1/10^3$ M	.015
	$5/10^3$ M	.014
	$1/10^2$ M	.014
K+ (Cl-)	$1/10^3$ M	.017
	$5/10^3$ M	.016
	$1/10^2$ M	.017
NH4+ (Cl-)	$1/10^3$ M	.013
	$5/10^3$ M	.015
	$1/10^2$ M	.016
Control	Tris-HCl buffer 0.05M pH 7.2	.016

b) Co-factors	Concentrations	DAT Activity (Units/min)
Pyridoxal Phosphate	$5/10^3$ M	Reacts with dopamine
Mercapto-ethanol	$5/10^3$ M	Reacts with DTNB
Dopamine	$1/10^3$.M	.0232
Control	Tris-HCl buffer 0.05M pH 7.2	.0310

0-3 day old adults were ground with Tris-HCl buffer, 0.05M, pH 7.2, containing $1/10^3$ M PTU and 10% glycerol in a proportion of 200 mg flies per ml buffer. Metallic ions or co-factors were added to the assay mixture. Assay conditions were similar to those described in Materials and Methods.

F) Partial Purification of DAT.

A partial purification of DAT was obtained by the following steps.

1) Ammonium sulfate fractionation.

The fractionation was performed on crude extracts (see Materials and Methods) from either third instar larvae or young adults (0-3 day old) by dropwise addition of a saturated solution of ammonium sulfate to the crude extract kept on ice. As shown in table 3, approximately 76% of the total DAT activity was found in the fractions containing 50 to 80% ammonium sulfate , and a 3 fold purification was obtained. The relatively low recovery after ammonium sulfate fractionation cannot be explained, but a similar problem was encountered during chromatography on Sephadex (see below).

2) Column chromatography.

I. DEAE column chromatography.

This chromatography procedure was unsatisfactory for DAT, since no enzyme activity could be recovered from columns prepared as described in the Materials and Methods. Since gradients of NaCl from 0.1 to 1.0 M and pH from 2.0 to 11.0 were ineffective in releasing the enzyme from DEAE, it is unlikely that an ionic adsorption was taking place. The possibility that the enzyme was inactivated by DEAE

TABLE 3. Ammonium sulfate fractionation.

% saturation	Distribution of DAT activity
0-25%	1.1%
25-40%	5.7%
40-50%	11.8%
50-60%	18.2%
60-70%	42.5%
70-80%	16.3%
80-90%	4.4%

Recovery of 58% (based on total activity recovered). Cut used: 55-80%. Total recovery of 40% (3 fold purification).

The ammonium sulfate fractionation was performed by drop-wise addition of a saturated solution of ammonium sulfate (22° C) (buffered to 7.2 with NaOH) to a crude extract (0-3 day old adults in a proportion of 200 mg flies per ml Tris-HCl buffer, 0.05 M, pH 7.2) kept on ice.

The precipitate was separated by centrifugation (8,000 x G x 10 min) and redissolved in 2 x 0.25 ml Tris-HCl buffer, 0.05 M, pH 7.2. The solution obtained was not dialyzed since ammonium sulfate did not interfere with enzyme activity.

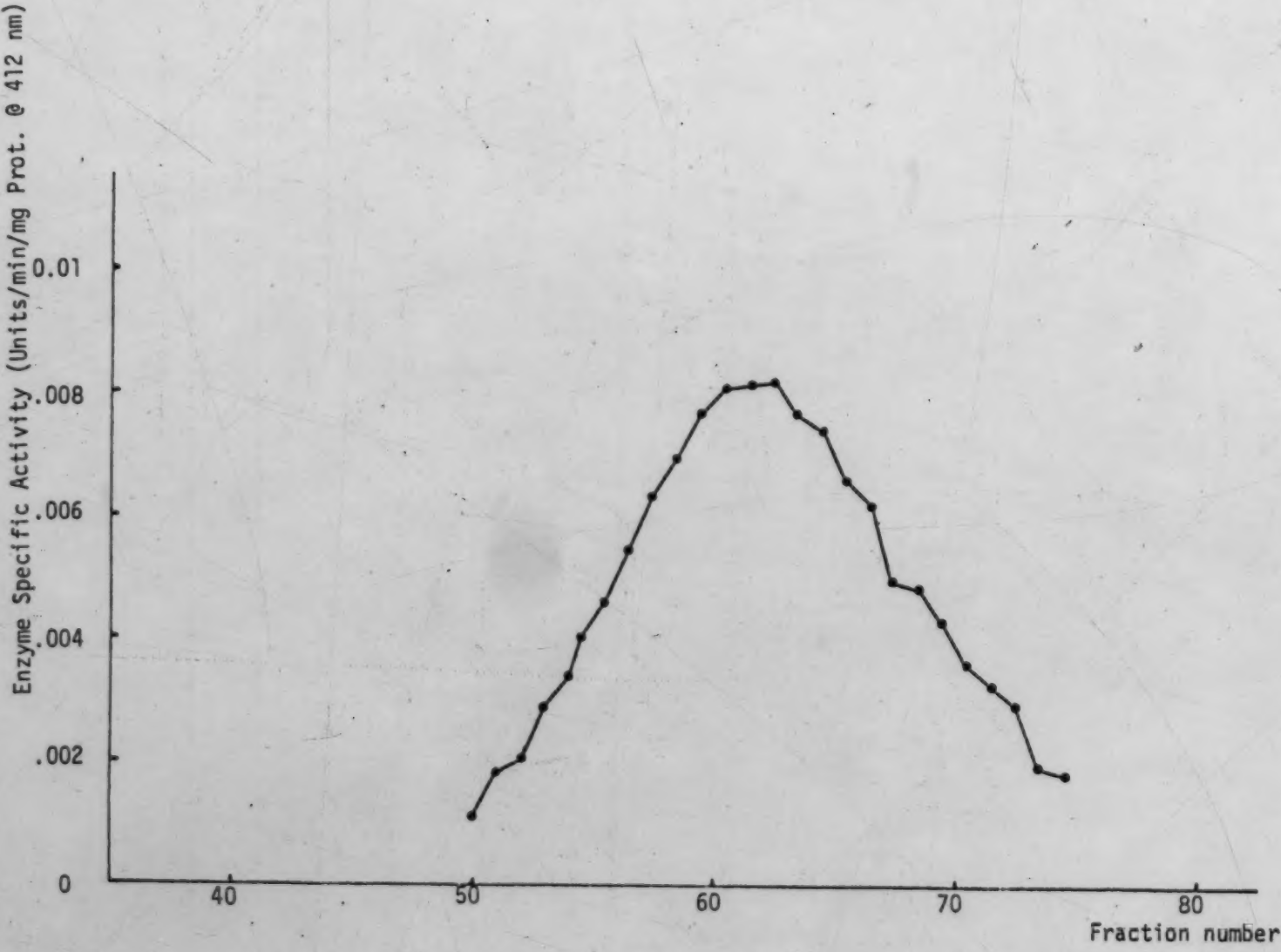
was not pursued, and our investigation of the chromatographic behavior of the enzyme on DEAE was terminated at this point.

II. Sephadex G-200 and G-150 column chromatography. Chromatography on Sephadex G-200 or G-150 proved to be a useful technique for obtaining a purification. A typical profile of DAT elution on Sephadex G-200 is illustrated in figure 13. As the figure shows, most of the activity eluted between fractions 57 and 68. When the peak fractions were pooled, typically a 3.4 fold purification was obtained with a recovery of 63%. Similar results were obtained on Sephadex G-150 since the molecular weight of the enzyme (approximately 28,000 daltons ,see later) is substantially below the exclusion limit for both types of gel. While the relatively long period of time (4-5 hours) required to elute these columns might be consistent with recoveries observed, a surprisingly low recovery (60-70%) was also observed when crude extracts were passed over small (1.2 X 15 cm) Sephadex G-25 columns. If an aliquot (0.5 ml) of a crude extract containing a known concentration of activity was mixed with 5 ml of pre-soaked Sephadex G-25, G-150 or G-200 in a beaker, no significant loss of enzyme activity was observed after 30 minutes at 4°C. Hence, the enzyme is not inactivated by

Fig. 13. Elution profile of DAT on Sephadex G-200.

Chromatography on Sephadex G-200 was performed on a 1.2 x 100 cm column using a peristaltic pump with a flow rate of 10 ml/hr. 1.0 ml of partly purified crude extract (after ammonium sulfate fractionation) was loaded on the column.

The chromatography was done in the cold (4° C) and 20 drop fractions (averaging 0.9 ml) were collected. Enzyme activity was measured by the procedure described in Materials and Methods, and protein determination was done according To Lowry et al. (1951).


Enzyme Specific Activity (Units/min/mg Prot. @ 412 nm)
0.01
.008
.006
.004
.002
0
40
50
60
70
80
Fraction number

the beads nor does it adsorbs to the beads.

To determine whether or not the loss of a low molecular weight co-factor or activator was occuring during dialysis on G-25, the following experiment was performed. The fractions containing the DAT activity were intermixed with the pooled remaining fractions (fig. 14). After correcting for the dilution, no increase in enzyme activity could be detected in any of the pooled samples. In accord with this observation is our finding (to be discussed later) that there are no apparent co-factor requirements of the enzyme. Pre-treatment of a G-25 column (1.2 X 15 cm) by passage of 10 ml of a BSA (bovine serum albumin) solution (5 mg/ml) also failed to improved recovery. Since an analysis of the stability of the enzyme at 4°C (see later) revealed no significant loss of activity during a 3 to 4 hour period, we have no satisfactory explanation for the low recovery from Sephadex at the present time.

3) Affinity Chromatography.

Due to difficulties encountered in obtaining good recovery after standard purification procedures (column chromatography and ammonium sulfate fractionation), the relatively new technique of affinity chromatography was

Fig. 14. Sephadex G-25 dialysis.

1.0 ml crude extract
to
Sephadex G-25 column
to
Fractions collected: I - 0 to 5 ml
II - 5 to 10 ml
III - 10 to 15 ml
IV - 15 to 20 ml

Activity in combined or separated fractions:

Fraction#	Activity (corrected for dilution)
I	0%
II	0%
III	100%
IV	0%
I & II	0%
I & IV	0%
II & IV	0%
III & I	100%
III & II	100%
III & IV	100%

1.0 ml of crude extract from 0-3 day old adults (in a proportion of 200 mg flies per ml Tris-HCl buffer, pH 7.2, 0.05 M) was loaded on a pre-equilibrated Sephadex G-25 column (1.2 x 12 cm). Approximately 70% of the total activity loaded on the column was recovered in fraction III (10 to 15 ml). This accounted for 100% of the recovered activity. Intermixing of the fractions revealed no increase or decrease in activity. Assay conditions were similar To those described in Materials and Methods.

tried.

The principles for affinity chromatography were first described by L.S.Lerman, in 1953:

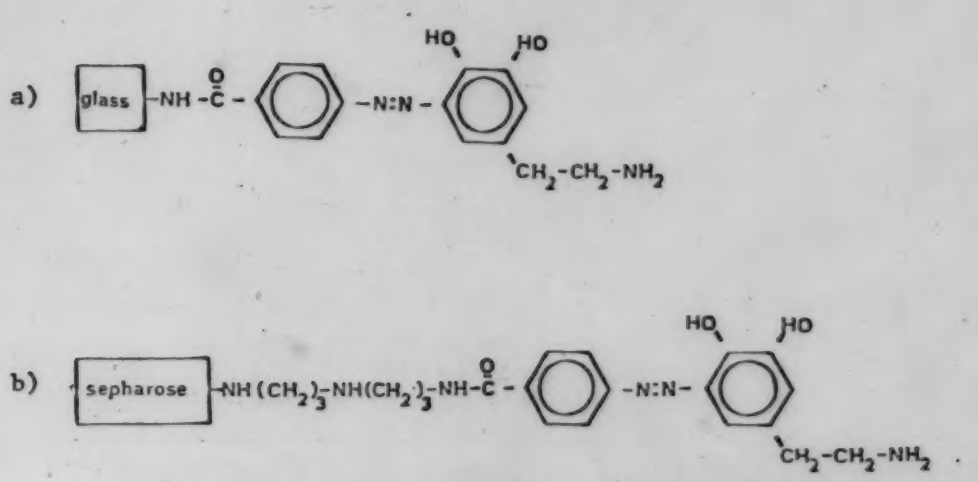
> If a specific competitive inhibitor or a suitable substrate of an enzyme is coupled to the surface of an inert solid, it may be expected that the enzyme will be reversibly bound to the solid through combination with the attached group, while enzymes with different specificities and other proteins will not be bound. The enzyme could later be released by a change in the medium to conditions unfavorable for combination as, for example, change in pH or salt concentration. Alternatively, by the use of a solution of a competitive inhibitor, the enzyme, will, according to the principle of mass action, be returned into solution by an additional specific process. Thus, the isolation of the enzyme would depend directly on its catalytic specificity rather than, as in

conventional procedures, its properties as a protein.

The technique was not developed, however, for 15 years. By this time, appropriate adsorbents had become available. Of interest to us was the development of a technique to bind catecholamines (noradrenaline in particular) to porous glass beads (Venter, 1972). Since the structure of noradrenaline differs from that of dopamine only by the presence of an additional hydroxyl group on the side chain, and since the binding of noradrenaline to the matrix was through the benzene ring, we expected that dopamine might also be attached to a glass matrix. Purification of DAT might then be effected through specific binding of the enzyme to the substrate. In order to facilitate such binding, the substrate is usually not attached directly to the beads. Rather, it is covalently bound to one end of a 5-6 carbon chain, the other end of which is bound to the matrix. A schematic representation of the dopamine attached to the matrix is shown in figure 15a for the glass matrix while the Sepharose matrix is shown in figure 15b.

Attachment of dopamine to aryl amine glass beads (as described in chapter 2) was monitored by the fixation of

Fig. 15. Attachment of dopamine to: a) glass matrix
b) Sepharose matrix



GAO-3940 porous glass beads with an average pore diameter of 550 Angstroms (Corning Glass) and Sepharose 4B (Pharmacia Fine Chemicals) were treated as described in Materials and Methods which resulted in the fixation of dopamine to their surface through an intermediate coupling molecule.

^{14}C-dopamine to the glass beads. The amount bound, 0.680 mg per 1.0 gm of beads, compared favorably to the attachment of adrenaline to glass beads, which resulted in a fixation of 0.193 mg per gm of glass beads (Venter, 1973). A non-specific adsorbtion of ^{14}C-dopamine was observed to untreated glass beads. However, this amounted to only 7.2% of the specific absorbtion observed when beads were treated as described in Materials and Methods.

A small column (0.5 X 6.0 cm) of the glass matrix was packed and loaded with crude extracts (from 0 to 3 day old adults) and eluted with

1) Buffers of different pH (1.0 to 11.0) and molarity (.05 to 0.5 M)

2) Various salt concentrations (0.1 to 1.0 M)

3) Relatively concentrated solutions of dopamine (5 mg/ml) and acetyl CoA (2 mg/ml)

No enzyme activity was recovered under any of these conditions and 80% of the protein present in the extract was retained on the column due to non-specific adsorbtion by the porous glass beads. To eliminate this problem the column was loaded with 5.0 ml of a solution of BSA (5.0 mg/ml) in buffer. This treatment prevented the majority

of the protein in the crude extract from being retained but unfortunately it also prevented the binding of DAT to the matrix .

In an attempt to surmount these difficulties, Sepharose, an adsorbent having little affinity for protein (Cuatrecasas, 1970, 1971), was tried as a support. Furthermore, a longer intermediate chain was used to attach the ligang to the solid matrix (fig. 15b) to minimize any interaction between the enzyme and the Sepharose. Using the procedure described in chapter 2, 0.36 mg of dopamine was attached per gram of Sepharose.

A wide variety of conditions were tried in an unsuccessful attempt to elute the enzyme from the column. These included:

Borate buffer at pH 10.0, 0.1 M

KCl-HCl buffer at pH 2.0, 0.0125 M

Concentrated substrate solutions; either

dopamine (5.0 mg/ml) or

acetyl CoA (2.0 mg/ml) in Tris-HCl buffer, pH 7.2, 0.05 M.

An attempt to remove the enzyme by electrophoresis on column was also unsuccessful.

When cclumns loaded with an excess of crude extract (2.0 ml or more for a 0.5 X 6.0 cm column) were eluted with Tris-HCl 0.05 M, pH 7.2 containing 1.0 M NaCl, 97 % of the DAT retained on the beads was recovered, with a 3.6 fold increase in specific activity. This indicates that many proteins in addition to DAT were retained on the column. Using a gradient of NaCl (0.1 to 1.0 M) to elute the cclumn improved the purification to 7.5 fold (fig.16a), while a pH gradient (6.5 to 8.5) gave a 24 fold increase in specific activity (fig. 16b) if the peak fractions were pooled. It was not possible to improve the recovery or to increase the purification by the following procedures:

1) Pretreatment of the column with BSA (5.0 mg/ml)

2) Elution at lower speed (1 drop/min from 3 drops/min)

3) Operation at a different temperature (4°C from room temperature)

4) Elution with buffers of different molarities (0.01 to 0.5 M) and different pH values (2.0 to 10.5).

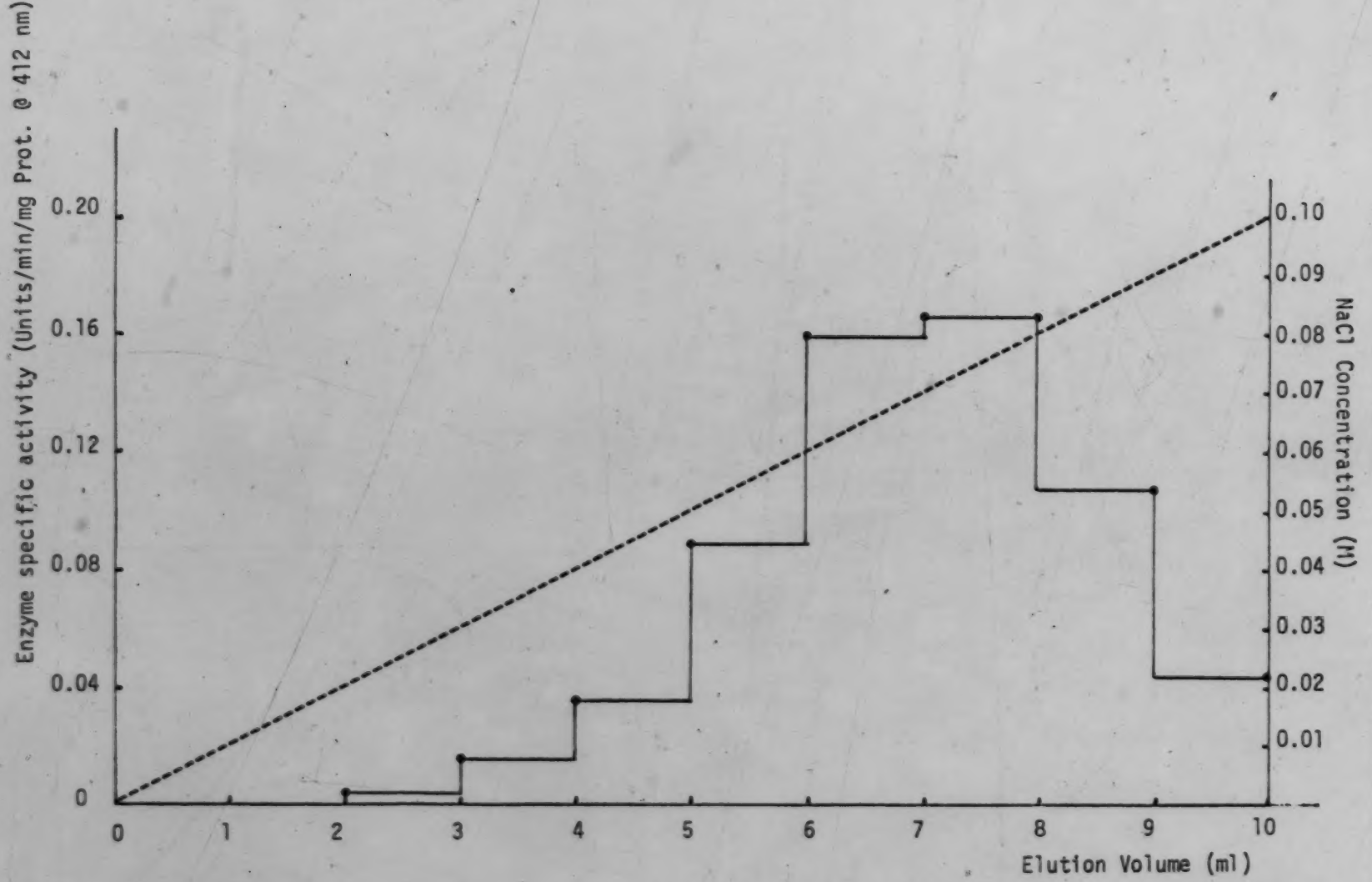
It could well be argued that these results indicate

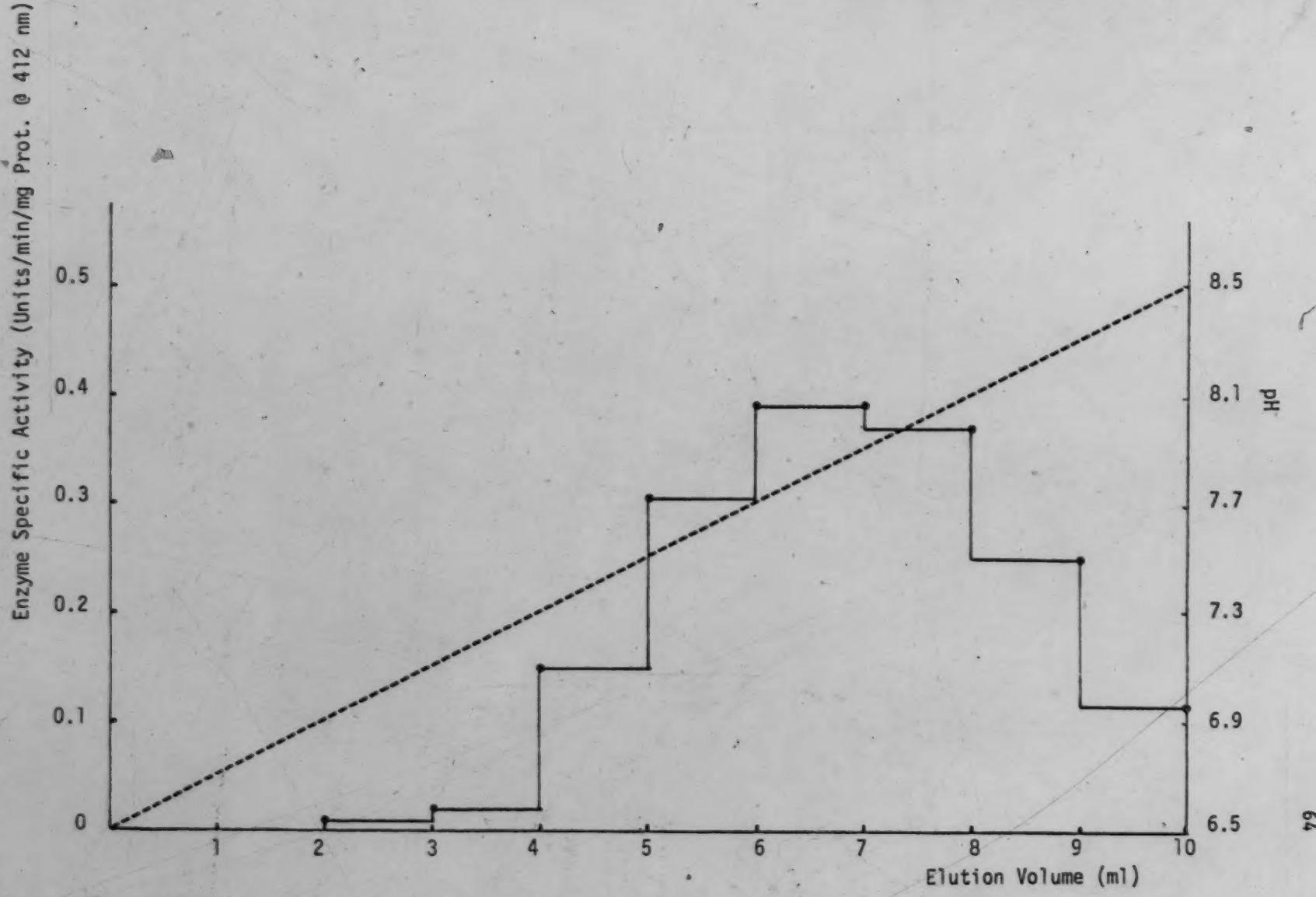
Fig. 16a and b. Chromatographic behavior of DAT on a Sepharose affinity column.

a) Elution with a gradient of NaCl

b) Elution with a gradient of pH

2.0 ml of crude extract was loaded on a 0.5 x 6.0 cm column containing Sepharose beads with attached dopamine. The column was pre-eluted with Tris-HCl buffer, 0.05 M, pH 7.2 and the total activity eluted determined. By calculation, an estimation of the activity remaining on the column was made. The column was then eluted with, Fig. 16a, a gradient of NaCl (0.1 to 1.0 M) in Tris-HCl buffer, or, Fig. 16b, a pH gradient (Tris-maleate, 0.05 M). Assay conditions were similar to those described in Materials and Methods. Protein determination was based on the method of Lowry (1951).


Enzyme specific activity (Units/min/mg Prot. @ 412 nm)
0.20
0.16
0.12
0.08
0.04
0
0
1
2
3
4
5
6
7
8
9
10
Elution Volume (ml)
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
NaCl Concentration (M)


Enzyme Specific Activity (Units/min/mg Prot. @ 412 nm)
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
9
10
Elution Volume (ml)
8.5
8.1
7.7
7.3
6.9
6.5
pH

the column was not functionning as an affinity column at all. We do not believe this is entirely the case since, if a new column is over-loaded with crude extract (for example 2.0 ml of crude extract on a 0.5 X 6 cm column), and eluted with Tris-HCl buffer, .05M, pH 7.2, the enzyme behaves in three different ways (fig. 17): 1- a good proportion (approximately 30 %) is retained on the column; 2- the majority (60 %) is eluted with most of the proteins (peak I) and 3- a small fraction (5 %) of the activity is eluted as a retarded peak (II). The DAT in peak II may be retarded by its specific affinity to the substrate attached to the matrix. Although the specific activity in this peak is very high, only a small portion of the enzyme present in a crude extract is contained in this peak. However, the use of a much larger column might allow one to increase the recovery to a point where this could become a useful purification step.

In conclusion, it appears that affinity chromatography could be employed to obtain a purification of DAT, although the results are disappointing in the light of other reports (Miller, 1972; Cuatrecasas, 1968; Wilcheck, 1969). As summarized in table 4, a combination of ammonium sulfate fractionation, column chromatography on Sephadex and affinity chromatography might be expected

Fig. 17. Elution of DAT and proteins on affinity chromatography columns.

2.0 ml of crude extract from 0-3 day old adults (in a proportion of 200 mg flies per ml Tris-HCl buffer, 0.05 M, pH 7.2) was loaded on a 0.5 x 6.0 cm column containing Sepharose beads with attached dopamine.

Elution was performed with Tris-HCl buffer, 0.05 M, pH 7.2, at room temperature. Enzyme activity and protein concentration were determined by the procedure described in Materials and Methods.

——— Enzyme activity

..... Protein concentration

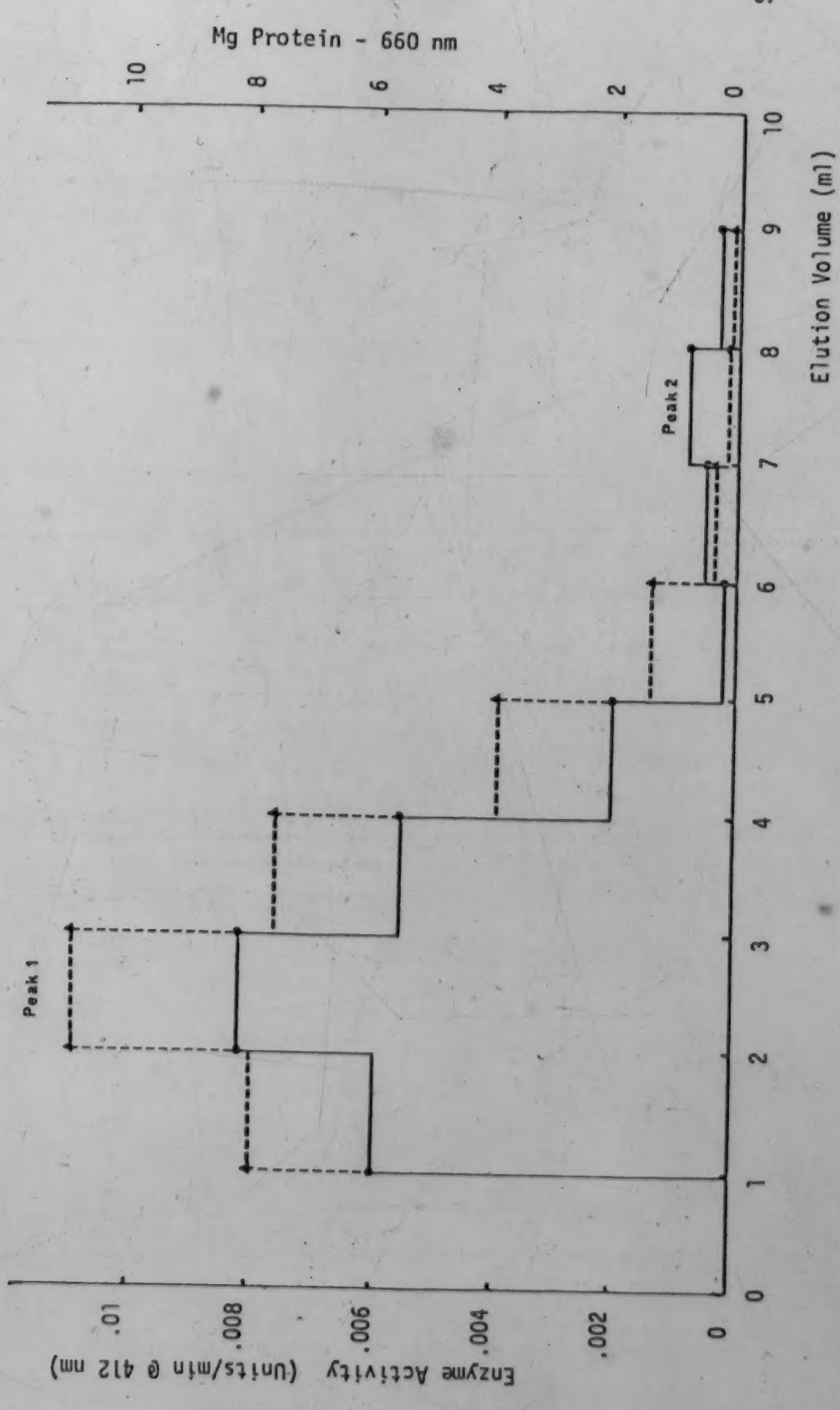

Mg Protein - 660 nm
10
8
6
4
2
0
Elution Volume (ml)
0
1
2
3
4
5
6
7
8
9
10
Peak 1
Peak 2
Enzyme Activity (Units/min @ 412 nm)
0
.002
.004
.006
.008
.01

TABLE. 4. Summary of purification steps for DAT.

	Final recovery %	Final purification x fold
crude extract	100	1.0
ammonium sulfate fractionation	44	3.2 (note 1)
Sephadex G-200 eluate	29	11.2/3.2=4 (note 2)
Affinity chromatography eluate	14	84/11.2=7.5 (note 3)

A crude extract prepared from 0-3 day old adults was used for all steps.

Note 1) - 50-75% cut

Note 2) - peak fractions pooled

Note 3) - NaCl gradient elution, peak fractions pooled.

to provide an 84-fold purification with a recovery of 14 %. Any attempt to purify a large quantity of the enzyme would be difficult in the light of the low recoveries but also to the enormous size of the affinity column to be employed.

G) Km studies.

Figures 18 and 19 present the data from which the Michaelis-Menten constants for both dopamine and acetyl CoA were calculated. Double reciprocal plots of 1/v vs 1/s (Lineweaver-Burk determination) were made from which apparent Km (dopamine) and apparent Km (acetyl CoA) were calculated to be 2.7 / 10^5 M and 2.5 / 10^5 M respectively.

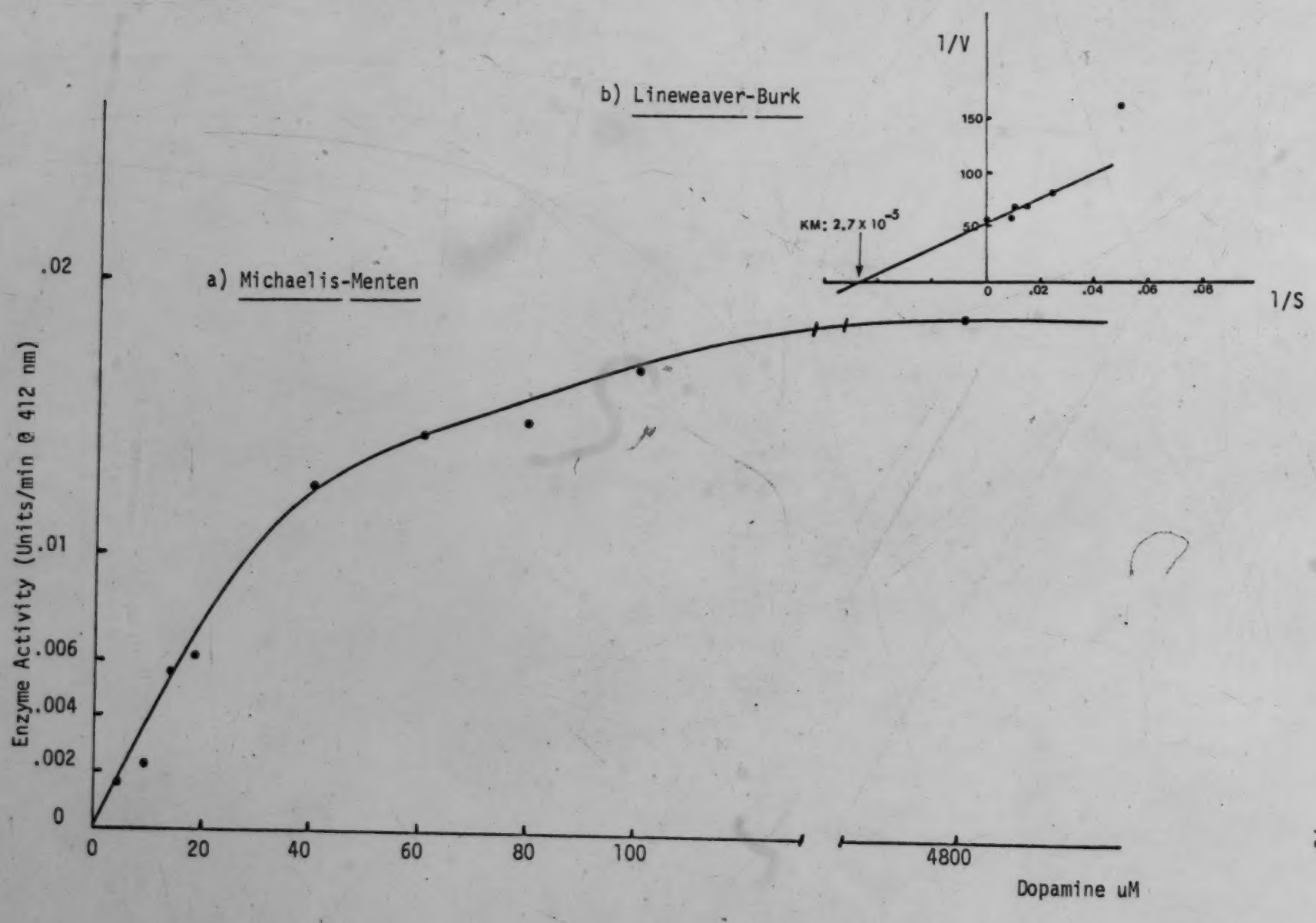
H) Estimation of the molecular weight.

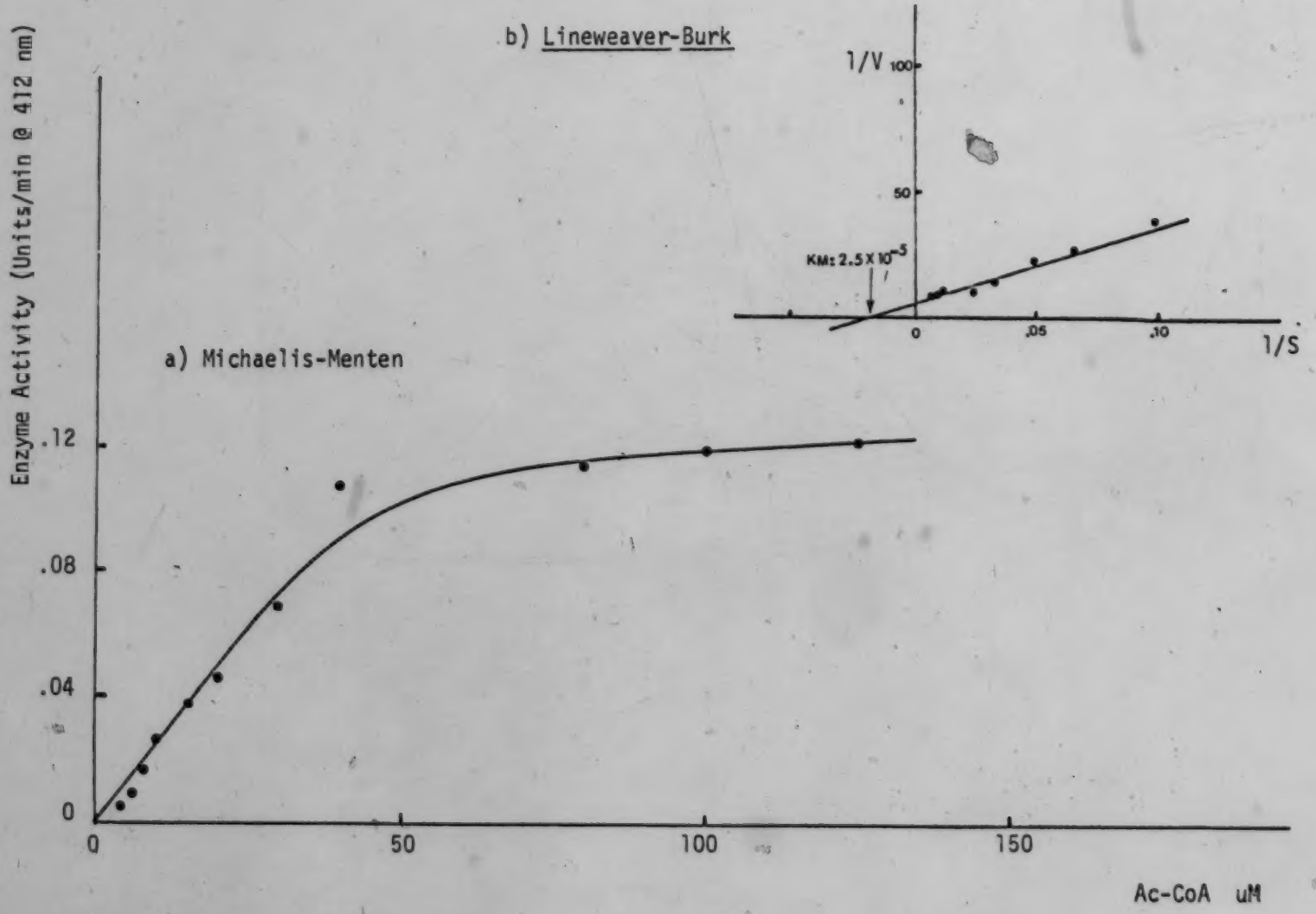
The apparent molecular weight of DAT was determined by gel filtration and verified by sucrose gradient centrifugation. As demonstrated by Andrews(1964, 1965), the elution of proteins on Sephadex gels is largely determined by their molecular weight. The molecular weight of a protein can be revealed by gel filtration even when present in a crude extract. Therefore only a simple partial purification is necessary to obtain reliable results.

Figs. 18 and 19. Estimation of apparent Km for dopamine and acetyl CoA.

The enzyme kinetics of DAT follow the Michaelis-Menten equation for both substrates, dopamine and acetyl CoA. Assay conditions were such that for the Km of dopamine, AcCoA was kept at a constant concentration (Km x 2) and the concentration of dopamine was varied. For the determination of the Km for AcCoA, the AcCoA concentration was varied while dopamine amount was kept constant (at Km x 2).

Concentrations are expressed as molarities in the assay mixture. A dialyzed crude extract from 0-3 day old adults was used for these studies.


a) Michaelis-Menten
b) Lineweaver-Burk
Enzyme Activity (Units/min @ 412 nm)
.02
.01
.006
.004
.002
0
0
20
40
60
80
100
4800
Dopamine uM
1/V
150
100
50
KM: 2.7 X 10^-5
0
.02
.04
.06
.08
1/S


b) Lineweaver-Burk
1/V
100
50
KM: 2.5 X 10^-5
0
.05
.10
1/S
a) Michaelis-Menten
Enzyme Activity (Units/min @ 412 nm)
.12
.08
.04
0
0
50
100
150
Ac-CoA uM

A Sephadex column (1.2 X 100 cm) was calibrated with 1.0 to 3.0 mg of each of the following standards:

- mycglobin (M.W. = 1.7 X 10^4 - 2 mg used)
- ovalbumin (M.W. = 4.5 X 10^4 - 1 mg used)
- bovine serum albumin (M.W. = 6.7 X 10^4 - 1 mg used)
- bovine gamma-globulin (M.W. = 1.6 X 10^5 - 3 mg used)

All protein standards were obtained from Sigma Chemical Co., and the molecular weight values were taken from the "Handbook of Biochemistry", 2nd edition, Chemical Rubber. The partition coefficients of each standard as well as DAT were calculated by the relationship Kav=(Ve-Vo)/(Vt-Vo), where Ve is the elution volume of the protein investigated, Vt is the total volume of the gel bed and Vo is the void volume. Kav ("K available") is related to the molecular weight of the proteins (Laurent and Killander, 1964). Figure 20 represents the chromatographic profiles of the protein standards on Sephadex G-200. As illustrated in figure 21, a linear relationship was observed when the Kav of each individual protein standard was plotted against its respective molecular weight on a semi-logarithmic scale. From 2

Fig. 20. Chromatographic behavior of DAT on Sephadex G-200.

Proteins in amounts varying from 1 to 3 mg (see text) were loaded separately on a Sephadex column (1.2 x 100 cm). Elution buffer (Tris-HCl, 0.05 M, ph 7.2) was provided through a peristaltic pump at a rate of 10 ml/hr. Protein estimations were made by the use of a UV spectrophotometer at 280 nm. DAT activity was measured as described in Materials and Methods.

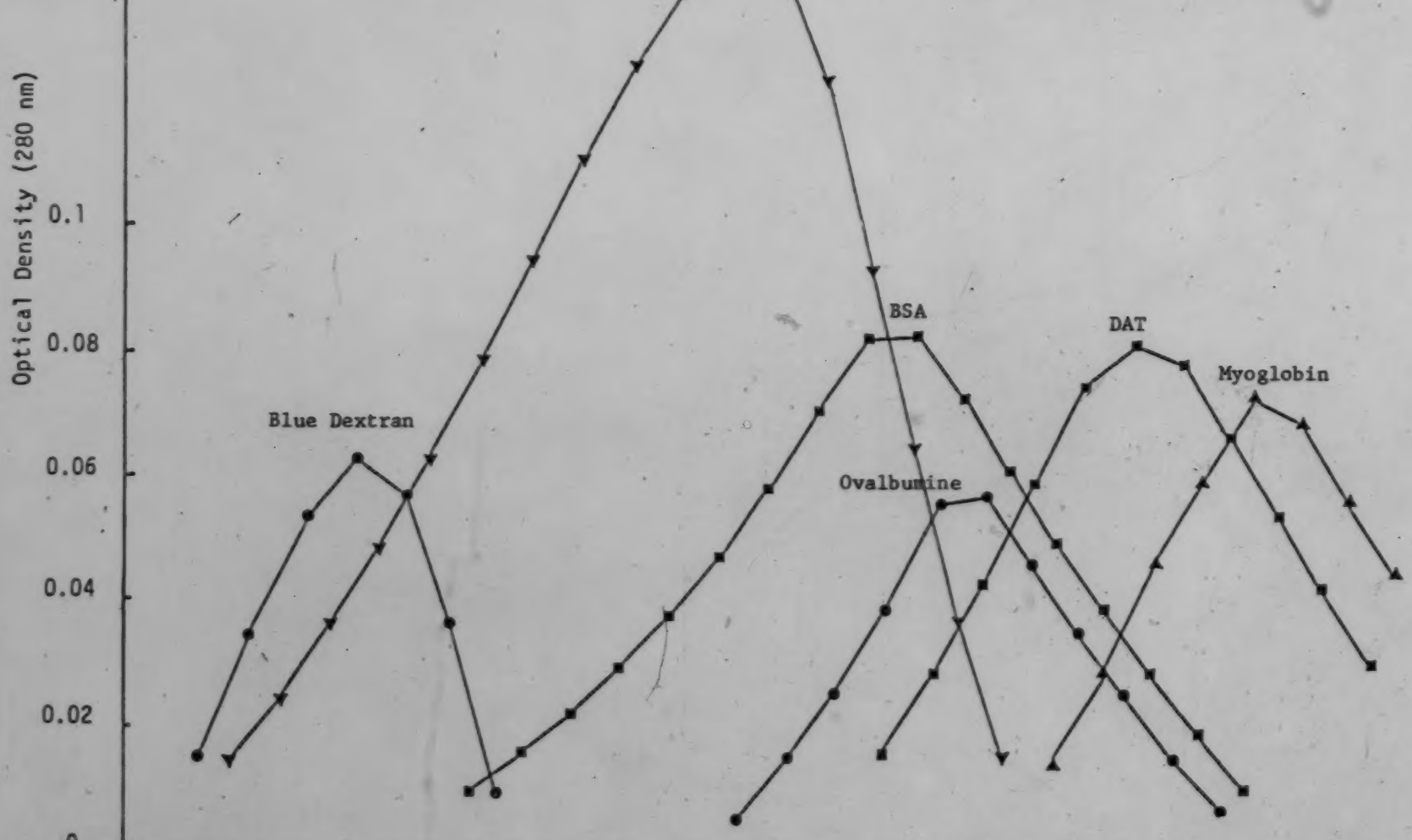

gamma-globulin
BSA
DAT
Myoglobin
Blue Dextran
Ovalbumine
Optical Density (280 nm)
0.1
0.08
0.06
0.04
0.02
0
25
30
35
40
45
50
55
60
65
Fraction Number

Fig. 21. The relationship of Kav to molecular weight on Sephadex G-200.

The Kav (available) were measured by the equation Kav=(Ve - Vo)/(Vt - Vo) (Laurent and Killander, 1964), Ve being the elution volume of a particular protein, Vt the total volume of the gel and Vo the void volume.

K_{av}
.08
.06
.04
.02
0
myoglobin
DAT
ovalbumine
BSA
gamma-globulin
1x10^3
2
3
4
5
6
7
8
9
1x10^4
.2
3
4
M.W. x 10^5

different runs on G-200, the apparent molecular weight of DAT was determined to be 2.7(+ or - 0.1) X 10^4 (fig. 21). The same procedure was repeated with Sephadex G-150 (fig. 22 and 23) and a molecular weight estimation of 3.2 X 10^4 was made.

Sucrose gradient sedimentations were performed according to Martin and Ames (1960) and the details are provided in the legend to figure 24 . The molecular weight estimate obtained from 2 runs was 2.9(+ or -0.2) X 10^4, in excellent agreement with the results from the gel filtration (fig. 25).

Fig. 22. Chromatographic behavior of DAT on Sephadex G-150.

See legend to Fig. 20.

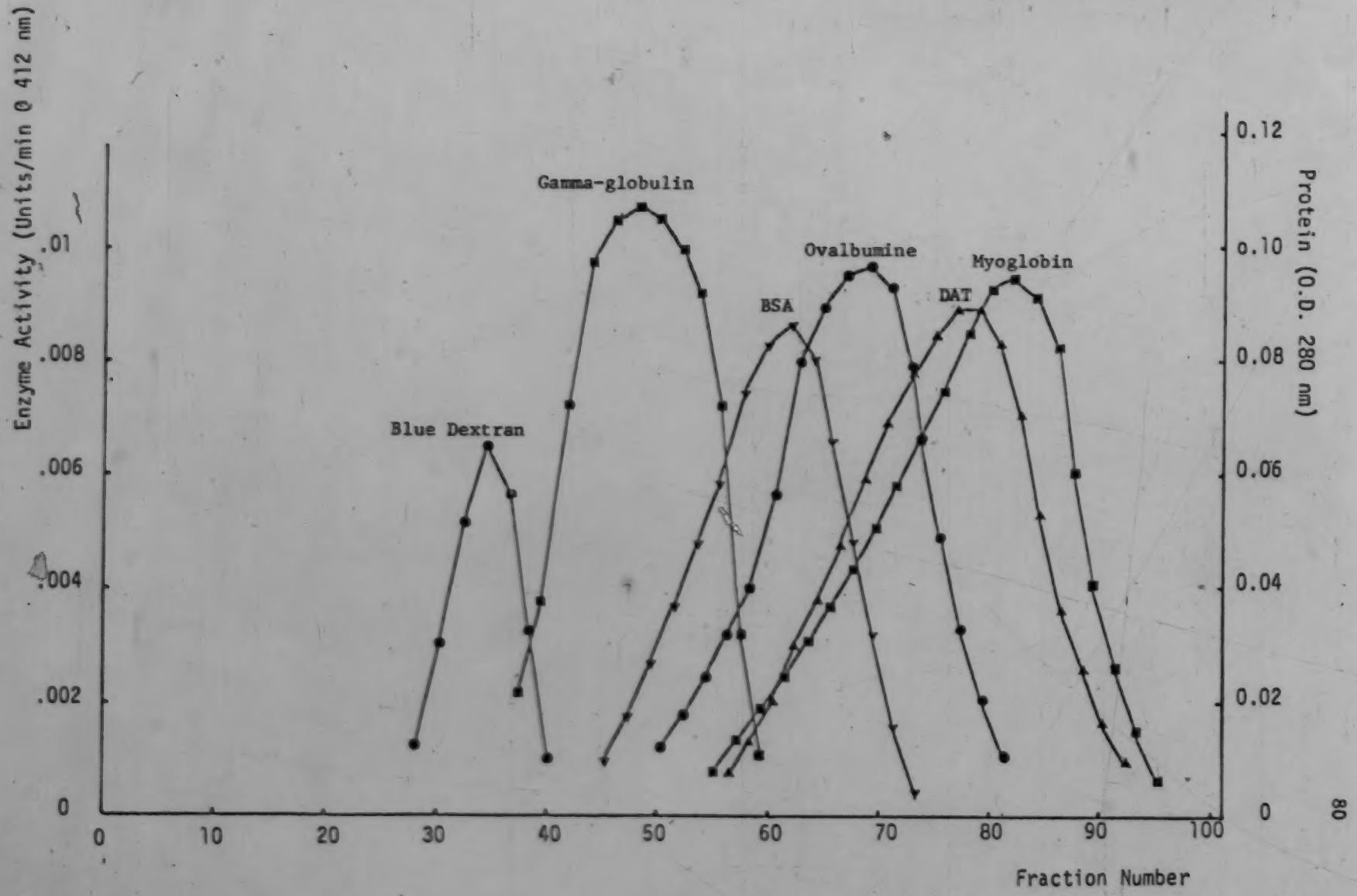

Enzyme Activity (Units/min @ 412 nm)
.01
.008
.006
.004
.002
0
Protein (O.D. 280 nm)
0.12
0.10
0.08
0.06
0.04
0.02
0
Gamma-globulin
Ovalbumine
Myoglobin
BSA
DAT
Blue Dextran
0
10
20
30
40
50
60
70
80
90
100
Fraction Number

Fig. 23. The relationship of Kav to molecular weight on Sephadex G-150.

See legend to Fig. 21.

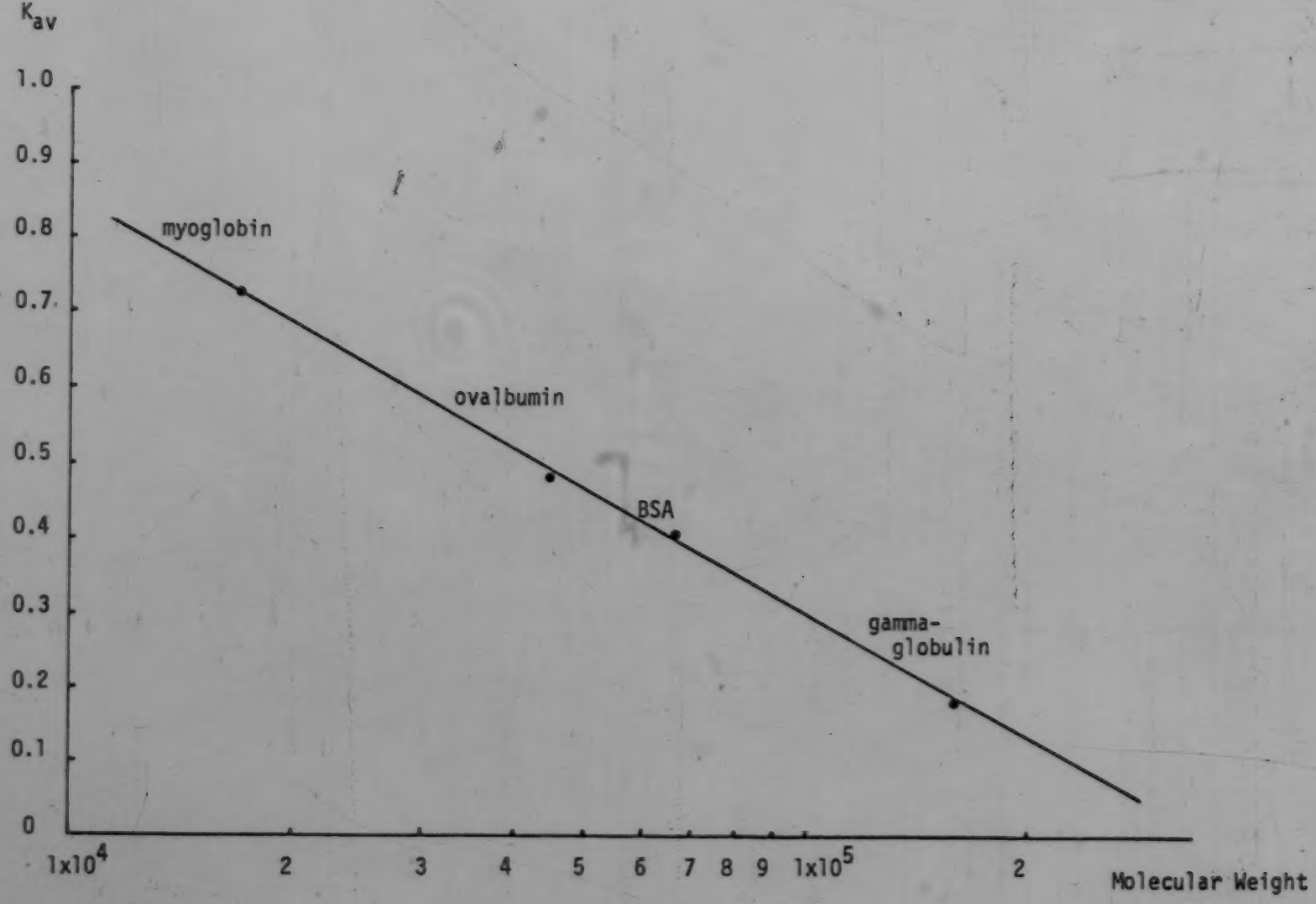

K_{av}
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
myoglobin
ovalbumin
BSA
gamma-
globulin
1x10^4
2
3
4
5
6
7
8
9
1x10^5
2
Molecular Weight

Fig. 24. Sucrose gradient sedimentation of DAT.

Proteins in amounts of 2 mg were layered on a 4.6 ml sucrose gradient (5 to 20%) and centrifuged at 40,000 RPM for 18 hours in a Spinco SW50.1 rotor. Fractions were collected by puncture at the bottom of the tube. Protein determination was done at 280 nm in a Beckman DB UV spectrophotometer.

2.0 ml of crude extract, prepared by grinding 400 adults in 2.0 ml of Tris-HCl buffer, were further purified with ammonium sulfate fractionation before layering on the sucrose gradient. DAT activity was measured as stated in Materials and Methods.

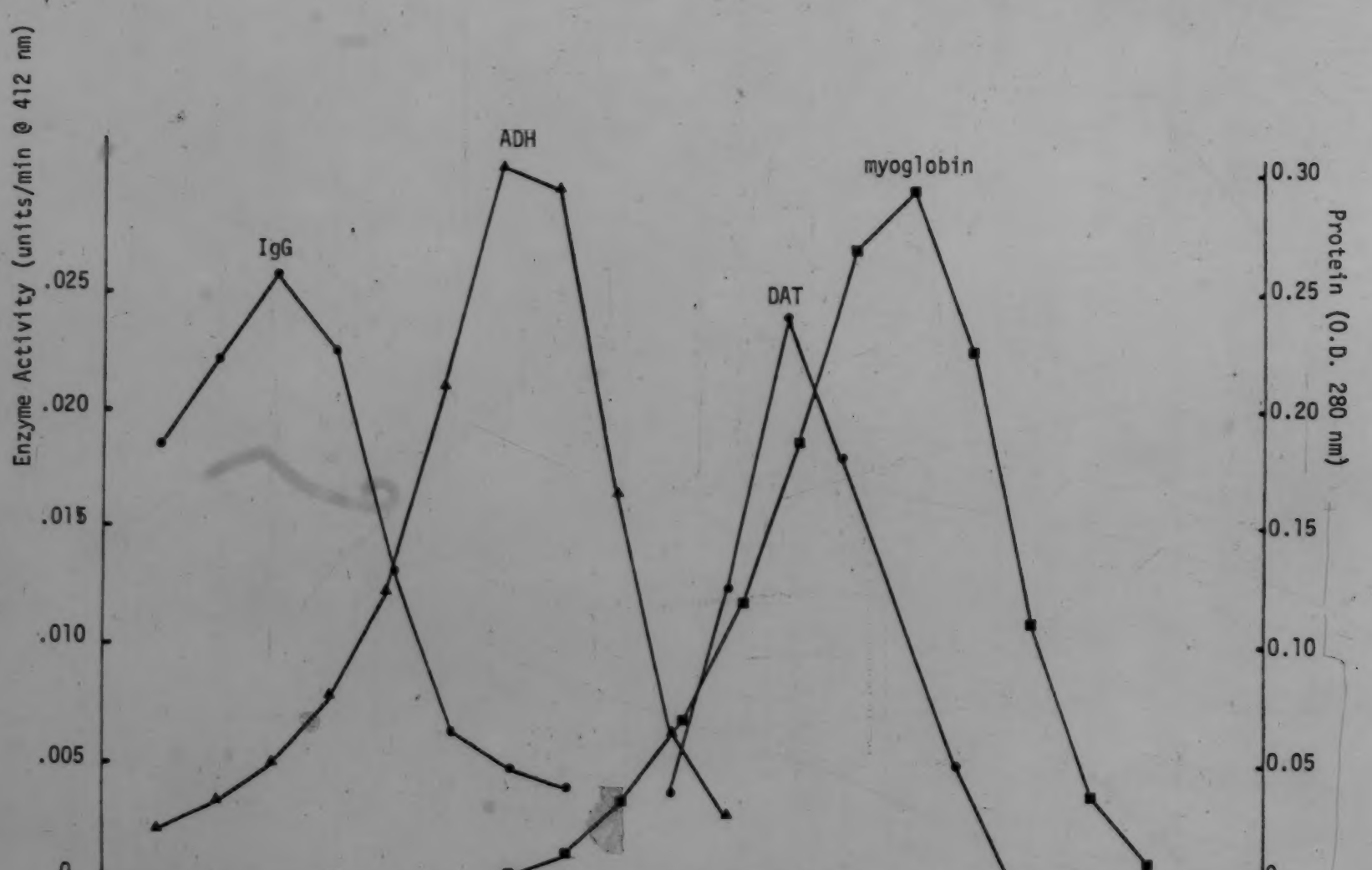

Enzyme Activity (units/min @ 412 nm)
.025
.020
.015
.010
.005
0
IgG
ADH
DAT
myoglobin
0.30
0.25
0.20
0.15
0.10
0.05
0
Protein (O.D. 280 nm)
0
1.0
2.0
3.0
4.0
Volume (ml)

Fig. 25. Estimation of the molecular weight of DAT from the sucrose sedimentation analysis.

The calculations were performed as stated by Martin and Ames (1960).

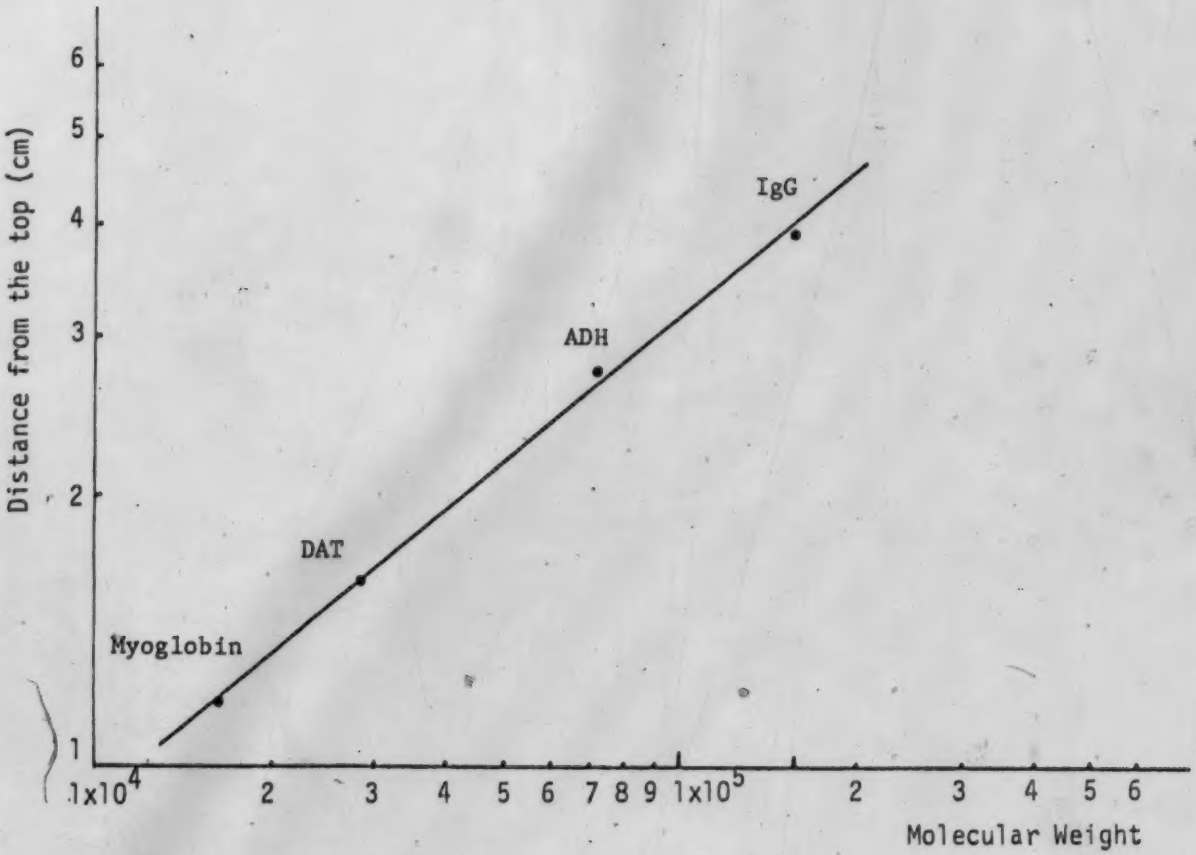
Distances sedimented were calculated from the mid-point of the layered sample to the mid-point of the peak of the protein.

Distance from the top (cm)
6
5
4
3
2
1
IgG
ADH
DAT
Myoglobin
1x10^4
2
3
4
5
6
7
8
9
1x10^5
2
3
4
5
6
Molecular Weight

Fig. 25. Estimation of the molecular weight of DAT from the sucrose sedimentation analysis.

The calculations were performed as stated by Martin and Ames (1960).

Distances sedimented were calculated from the mid-point of the layered sample to the mid-point of the peak of the protein.


Distance from the top (cm)
6
5
4
3
2
1
IgG
ADH
DAT
Myoglobin
1x10^4
2
3
4
5
6
7
8
9
1x10^5
2
3
4
5
6
Molecular Weight

CHAPTER 4

Biological and Genetical Studies.

A) Activity of DAT during development.

The DAT activity was measured in crude extracts prepared from different stages of development, from first instar larvae to 10 day old adults. As illustrated in figure 26, enzyme activity increased sharply during the late third instar, reached a maximum at pupariation and then decreased abruptly. A second increase began just prior to eclosion. The DAT activity remained rather high in the adults, representing about 2/3 of the maximum activity present at pupariation.

B) Tissue localization.

Experiments were conducted to measure the activity of DAT in different tissues and organs of *Drosophila* . These experiments were performed on third instar larvae; the location of DAT in tissues of adults flies has not been attempted. The first step in these experiments consisted of separating the interior organs from the cuticle. White puparia were sliced along the ventral mid-line and the internal organs were freed from the body wall with a scalpel. As indicated in table 5, the specific activity

Fig. 26. DAT activity during development at 25° C.

Insects at different stages of development were used to prepare crude extracts in a proportion of 200 mg flies per ml Tris-HCl buffer, 0.05 M, pH 7.2. The crude extracts were dialyzed on a 1.2 x 12 cm Sephadex G-25 column prior to assaying.

Assay conditions were similar to those described in Materials and Methods. Protein determination was by the method of Lowry et al. (1951).

The age corresponds to time after egg deposition.

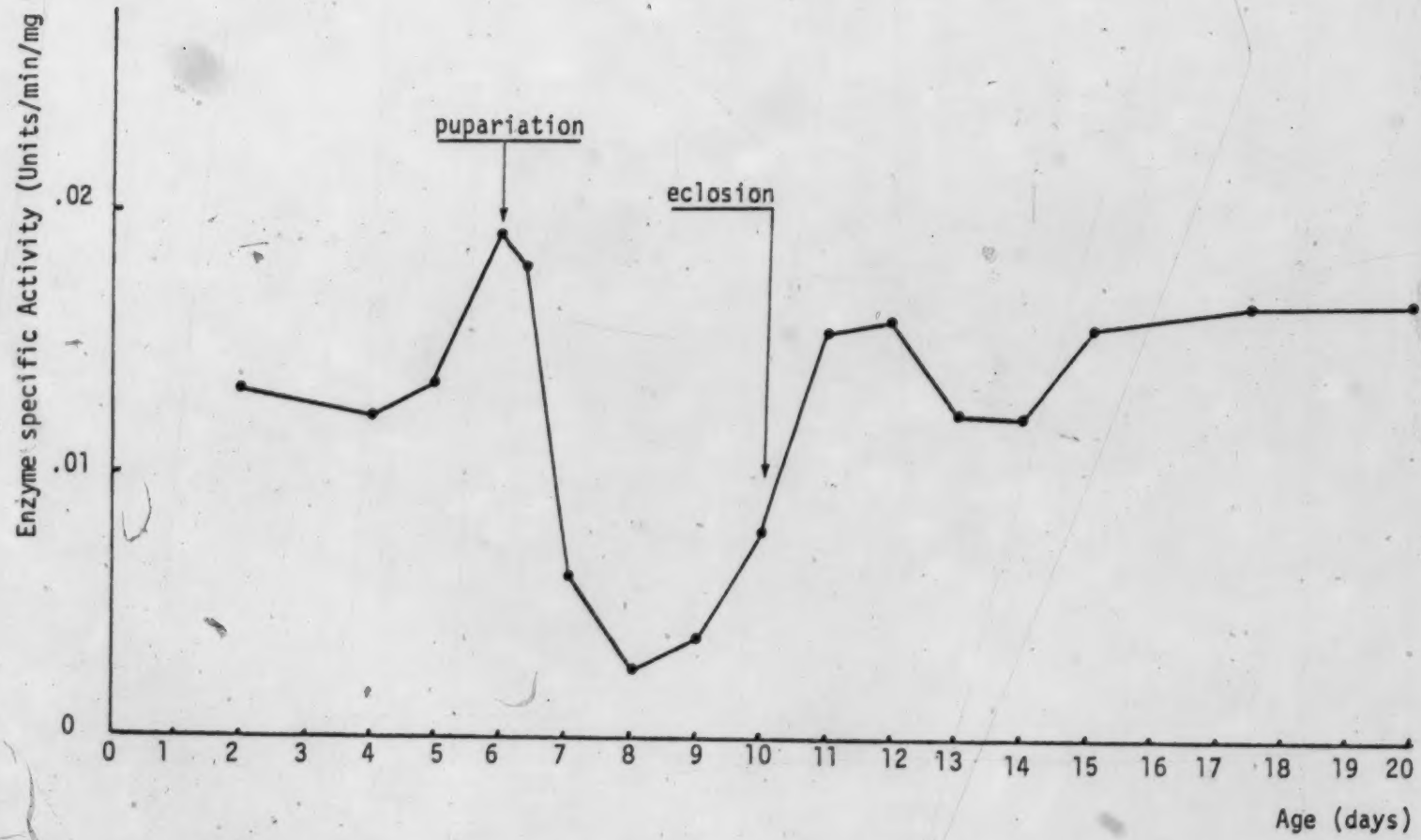

Enzyme specific Activity (Units/min/mg @ 412 nm)
.02
.01
0
pupariation
eclosion
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (days)

TABLE. 5. Localization of DAT in the organism.

	DAT		Dopa decarboxylase	
	Spec. Activity (Units/min/mg protein)	% of total Activity	Spec. Act. um Moles/20min/ mg protein	% of total Activity
Tissue				
Guts and organs	.0081	81.8%	7.21	32.6%
Cuticle	.0018	18.2%	14.90	67.4%

White puparium animals were used, sliced in the abdominal portion and the inside scraped out of the cuticle. DAT activity and protein concentration were determined as described in Materials and Methods. The measurements of dopa decarboxylase Were performed according to Chen (1974).

was 5 times as great in the inside organs than in the cuticle. This was a surprising observation and we felt initially that the epidermal cells had been scraped from the cuticle. To demonstrate that this did not occur, assays of dopa decarboxylase were carried out on the two fractions. The assays were performed according to Chen (1974) and these data (table 5) show clearly that in this preparation dopa decarboxylase specific activity in the inside organs was only 1/2 that in the cuticular fraction. These experiments forced us to accept the tentative conclusion that DAT, unlike dopa decarboxylase (Lunan and Mitchell, 1969; Chen, 1974) does not reside in the epidermal cells.

The next step was to determine the enzyme levels in a sample of the haemolymph. The haemolymph of 34 white puparia was collected with an elongated Pasteur pipette after puncturing the animals. The remaining bodies were ground and DAT activity measured in both extracts. About 7.5 times as much specific activity was found in the bodies compared to the haemolymph portion (Table 6a). In a modification of this experiments, 30 animals were punctured at both extremities, squeezed gently in Tris-HCl buffer containing PTU (.001 M) and assayed. No activity was observed in the solution in which the

TABLE 6. Determination of DAT in the haemolymph.

	DAT Specific Activity (Units/min/mg protein)	% of total Activity
A) Haemolymph	0.0130	11.7%
Bodies and inside organs	0.0990	88.3%
b) Buffer and haemolymph	0	0%
Bodies	0.0172	100%

In a) the haemolymph of 34 white puparia were collected and diluted in a 1:5 ratio with Tris-HCl buffer, 0.05 M, ph 7.2. The remaining bodies were ground in the same buffer containing 10% glycerol.

In b) 30 white puparia were gently squeezed after being punctured at both extremities, in Tris-HCl buffer, 0.05 M, pH 7.2. The remaining bodies were ground in this buffer containing 10% glycerol.

In both a) and b) assays and protein determination were performed as described in Materials and Methods.

animals were punctured and squeezed while a specific activity of 0.0172 units/mg wet weight/min was measured in the punctured bodies (table 6b). Finally, 50 animals, at the white puparium stage, were punctured, placed in Tris-HCl buffer, covered with sand and centrifuged at 9625 X g for 15 minutes. The sand was present to compress the animals under the centrifugal force and to expel the haemolymph into the supernatant. Here again, no activity was noticed in the supernatant while a specific activity of 0.110 units/mg wet weight/min was observed in the bodies. These experiments exclude the possibility that DAT is present in the haemolymph of Drosophila.

In a third series of experiments, 20 animals (white puparia) were dissected in Tris-HCl and the body walls, the muscles and internal organs (excluding the fat bodies) and the fat bodies were separated in three portions and washed with buffer. As shown in table 7, nearly as much activity was found in the internal organs as in the body walls, while nearly no activity was detected in the fat bodies.

In these experiments, it was particularly difficult to get a "clean" cuticular fraction and many muscle fragments remained attached to the body wall. To

TABLE 7. Distribution of DAT in tissues.

Tissue	DAT Specific Activity (Units/min/mg protein)	% of total Activity
Cuticle	.0200	42.5%
Wash portion	.0022	4.7%
Fat bodies	0	0%
Wash portion	.0019	2.3%
Organs and muscles	.0203	43.2%
Wash portion	.0035	7.4%

20 white puparia were dissected on ice in Tris-HCl buffer, 0.05 M, pH 7.2, the fat bodies, organs and muscles separated from the cuticle and washed with buffer to remove any enzyme that could have been present at the surface of the tissue. Enzyme activity and protein determination were Performed as described in Materials and Methods.

circumvent this problem, the experiment was repeated using white puparia from Sarcophaga bullata, which are 50 - 100 times the size of those from Drosophila. In this case, muscle fragments can be removed entirely from the body wall. We observed that most of the DAT activity was located in the muscle layer, which is closely attached to the cuticle at that stage (table 8).

In contrast, the majority of the dopa decarboxylase activity was found in the cuticle. This again Supports the contention that DAT and dopa decarboxylase are located in different tissues. We feel that these data suggest that DAT is located in oenocytes, large cells situated between the cuticular epidermal cells and the muscle layer, in contrast to dopa decarboxylase which is located in the epidermal cells. The observation that dopa decarboxylase activity was found in the muscle fraction (table 8) can be explained by contamination of this fraction by epidermal cells pulled away from the cuticle during dissection.

C) Genetic investigation on DAT.

I- DAT activity in mutants.

Abnormalities in dopamine levels in the mutant ebony, led us to examine the DAT activity in this mutant and the mutant black whose phenotype is very similar. As found

TABLE 8. Activity of DAT and dopa decarboxylase in various tissues of Sarcophaga bullata.

Tissue	Specific Activity			
	DAT (units/ min/mg Prot)	% of total Activity	Dopa decarbox. um Moles/20min/ mg protein	% of total Activity
a) cuticle	.0036	8.1%	2.25	44.4%
muscle	.0333	74.5%	2.65	53.0%
organs	.0078	17.4%	0.14	2.6%
b) cuticle	0	0%	3.16	52.1%
muscle	.0257	80.0%	2.62	43.2%
organs	.0064	20.0%	0.29	4.7%

White puparia from Sarcophaga bullata were dissected in Tris-HCl buffer, 0.05 M, pH 7.2 and DAT and dopa decarboxylase activity determined in the different fractions. In a) the animals had been kept at the white puparium stage at 4° C for 5 days prior to dissection. In b) fresh white puparia had been used. DAT activity and protein determination were as described in Materials and Methods. Dopa decarboxylase activity was measured As described by Chen (1974).

earlier by Hodgetts and Konopka (1973), no noticable difference was measured between e^{11} mutants and Canton-S wild type flies at pupariation. The same is true for black mutant, as illustrated in table 9.

However, in young adults (0 to 16 hours), only two-thirds the activity was present in ebony flies compared to the wild type (Canton-S), while slightly less than half the normal activity was measured in black (table 9). Some discussion of these observations will be given in the following chapter.

II- Attempted localization of the structural gene for DAT.

The correlation of a variation in enzyme activity with a variation in gene dosage has permitted the localization of several structural genes in Drosophila (Hodgetts, 1975; O'Brien and Gethmann, 1972). A technology for producing a series of non-overlapping, contiguous duplications has been developed in Drosophila (Lindsley et al, 1972), so that gene dosage effects can be studied throughout virtually the entire genome. The procedure used , including the designation of the stocks, is described by Hodgetts (1975). As the DAT activity is dependent on the stage of development, all the flies used

TABLE 9. DAT activity in mutants.

	DAT activity (units/min/mg wet weight)	
	White puparia	Young adults (0-16hrs old)
wild type (Canton-S)	.0224	.0161
ebony	.0207	.0122
black	.0197	.0075

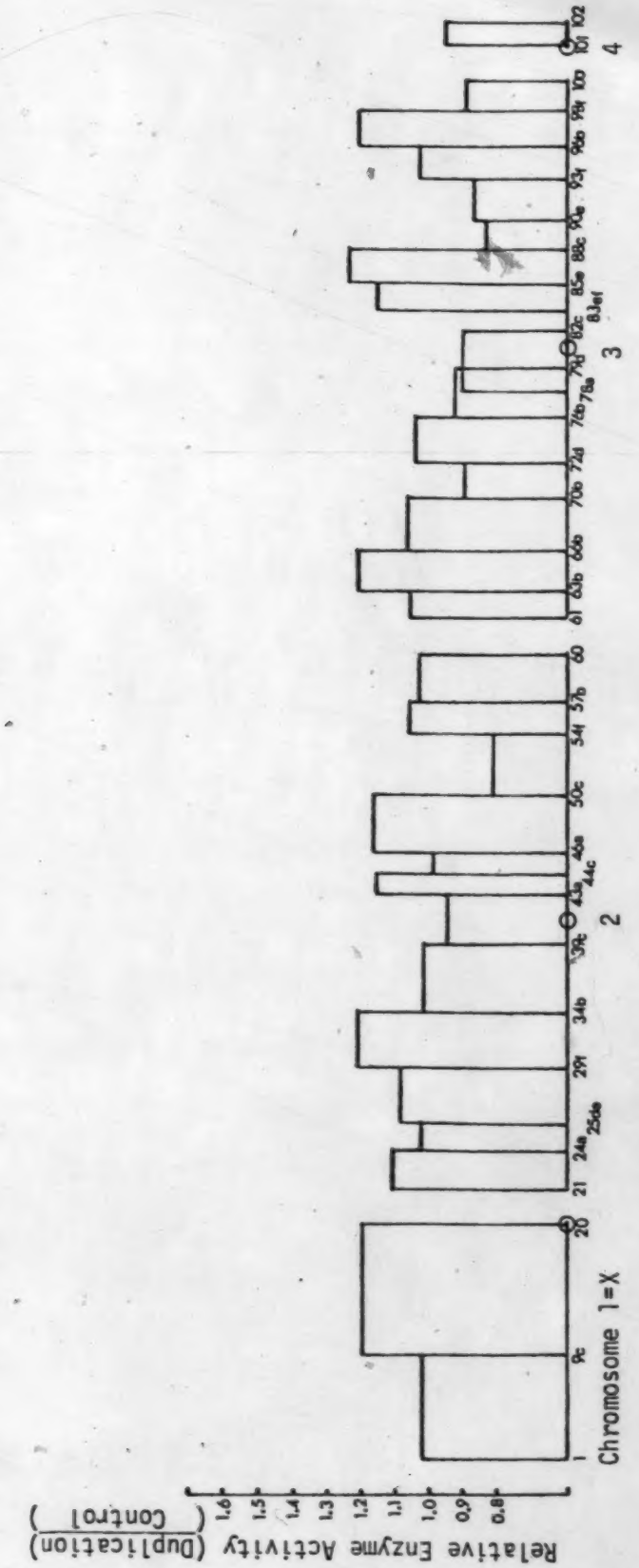
Crude extracts were prepared with animals at the white puparium and the adult stages by grinding the animals in Tris-HCl buffer, 0.05 M, pH 7.2, containing 10% glycerol. The enzyme activity was measured as described in Materials and Methods.

for this study were collected in a 4 hour period and aged for 26 + or - 2 hours. This insures that the enzyme activity is relatively high and constant (fig. 26). Where possible, 10 - 20 females were choosen for the DAT assay. In certain cases however, only 5 or 6 flies eclosed during the 4 hour collection period. However, the sensitivity of the assay is such that reliable data could be collected under these circumstances. The enzyme activity was always measured in duplicate extracts and two spectrophotometric estimates were obtained for every extract. The activity in the duplication-bearing aneuploids was then compared to that in sibling euploid sisters. In certain cases where an apparent difference (more than 20 %) existed between control and duplication-bearing flies, three and sometimes four measurements were made.The results which are shown in figure 27 also include the data for chromosomes 2 and 4 collected by Hodgetts (personnel communication). In no region of the genome was a 50 % increase in DAT activity associated with a duplication. Possible explanations for this result will be discussed in the following chapter.

Fig. 27. The response of DAT activity to increase gene dosage of the appropriate genotypes.

Flies were collected in a 4 hour period and aged for 26 + or - 2 hours. In most cases 10 to 20 flies were collected and then separated into two groups for preparation of crude extracts. Two enzymes assays were performed for each extract, resulting in at least a set of four DAT activity measurements for every section of the genome tested.

DAT activity was tested as described in Materials and Methods, and activity per mg of wet weight was calculated. The ratio activity in aneuploids/activity in euploids was determined and an average for every segment is shown in the figure.


Relative Enzyme Activity (Duplication / Control)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
Chromosome 1=X
1
9c
20
21
24a
25de
29f
34b
39c
43a
44c
48a
50c
54f
57b
60
61
63b
66b
70b
72d
74b
78a
79d
82c
84ef
85a
88c
90a
93d
96b
99d
100
101
102
2
3
4

CHAPTER 5

DISCUSSION

The preceeding pages describe some biochemical and biological properties of the enzyme dopamine acetyl transferase. We have developed a spectrophotometric assay for the enzyme, which permits a rapid determination of enzyme activity. While not as sensitive as the radiometric assay described by Dewhurst (1972), it has the advantage of being simpler.

Although the enzyme is not particularly easy to handle, reproducible results can be obtained if measures are taken to minimize denaturation. Of these, the most important were the inclusion of glycerol and albumin in the extraction buffers, and a short homogenization time. Our attempts to obtain procedures by which a purification of the enzyme might be affected were thwarted by low recoveries.If affinity chromatography was combined with ammonium sulfate fractionation and chromatography on Sephadex, a purification of one hundred fold could be expected with a recovery of about 10%. The rather low molecular weight of the enzyme (29,000) suggests that another purification procedure which separates molecules

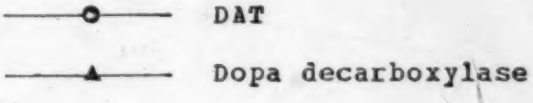
on the basis of size (eg preparative electrophoresis) might be successfully employed. Any attempt to obtain large amounts of a highly purified enzyme will be difficult unless some means to stabilize the enzyme during purification is discovered.

The developmental profile of DAT in Drosophila melanogaster showed some similarities to the dopa decarboxylase profile in this organism (McCaman et al,1972;Lunan & Mitchell,1969; Hodgetts,1975) and in Sarcophaga bullata (Chen,1974), having peak activities at pupariation and at eclosion. However, maximum activity for dopa decarboxylase slightly preceeds peak activity for DAT, this difference being more noticable at adult eclosion. As seen in figure 28, dopa decarboxylase peak activity occurs just before emergence of the adult fly and DAT approximately one day after adult eclosion. Interestingly, the DAT activity remains high in adults while dopa decarboxylase activity almost disappears shortly after emergence. This high level of DAT activity in adults could result from the fact that DAT is specific not only for dopamine, but also for tyramine and serotonin (Karlson & Ammon,1963; Dewhurst et al, 1972). N-acetylation could be an important mechanism for degradation of catecholamines, which may function as

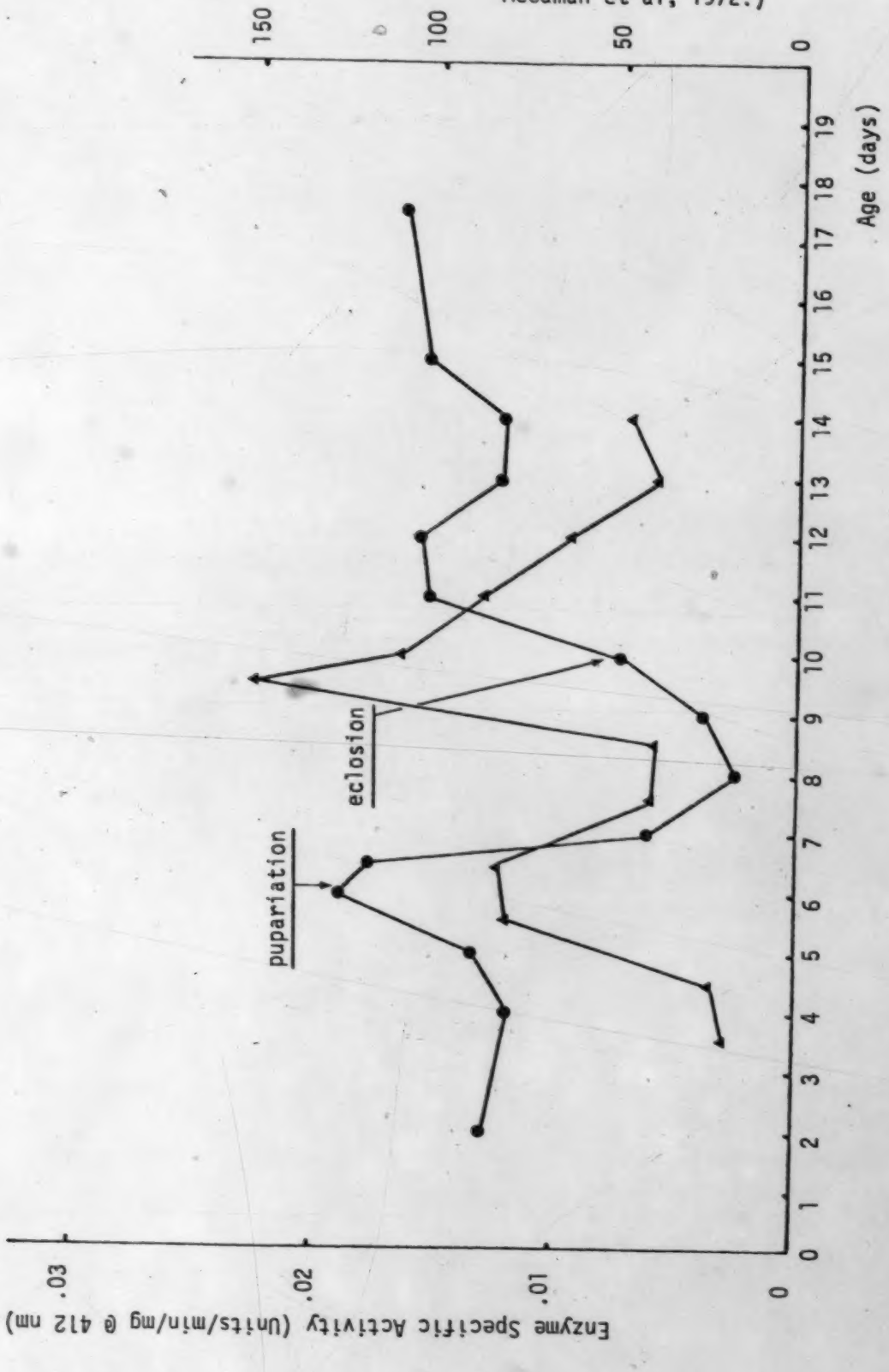
Fig. 28. DAT and dopa decarboxylase developmental profiles.

The data for DAT are taken from figure 26.

Dopa decarboxylase data were adapted from McCaman (1972).



Dopa decarboxylase Activity (u Moles/g protein/Hr. -
McCaman et al, 1972.)



neurotransmitters in insects (Oestlund, 1954; Molinoff & Axelrod, 1971). In fact, two enzymes involved in acetylcholine metabolism, choline acetyl transferase and acetyl cholinesterase, reach their maximum activities 8-30 hours after adult emergence in Drosophila (Dewhurst et al, 1970). Since DAT also peaks during this time, its involment in the metabolism of neurogenic catecholamines is possible. Obviously, further work would have to be undertaken to clarify this point. However, if large amounts of tyramine and/or serotonin are present after adult eclosion, these substrates would compete with dopamine for the enzyme. The transformation of dopamine would be reduced as a consequence, which could contribute to the relatively high level of dopamine present after eclosion (Hodgetts and Konopka, 1973).

One further point worth commenting upon can be made with reference to figure 28. As demonstrated by Hodgetts and Choi (1974), the dopamine level is high at pupariation and low at eclosion, which is consistent with the profile of DAT activity. This is the basis for the argument by these authors that, at eclosion, hardening in part occurs by the incorporation into the cuticle of indole quinones derived from the accumulation of dopamine. Excessive melanization is prevented by beta-

alanine which is postulated to form bridges between the indole quinones, thus minimizing the number of bonds between the indole quinones themselves. If beta-alanine metabolism is abnormal, as in the mutant black, dark body phenotypes are observed. At pupariation, by contrast, indole quinone formation never occurs due to the maintenance of low dopamine levels through N-acetylation. Thus not even the potential for excessive melanization exists at pupariation, in contrast to eclosion at which time dopamine levels are by comparison very high. Failure to detect mutants with black puparia is consistent with these notions. Another explanation of the formation of a light brown adult cuticle under conditions where the oxidation of the large reservoir of dopamine would lead to melanin formation is possible. As pointed out by Lunan & Mitchell (1969), dopamine could eventually be transformed into 3,4 dihydroxy-phenyl acetic which would behave as N-acetyl dopamine, forming brown pigments upon incorporation into the cuticle. The acetylation step would thus be bypassed and this would explain the sclerotization even under low acetylation conditions.

Juxtaposing the activity profile for DAT in Drosophila melanogaster upon the profile of the ecdysone titer in Calliphora erythrocephala (Shaaya &

Karlson,1965) and correcting for the difference in the time of development, as shown in figure 29, reveals several interesting facts (unfortunately, extensive data on ecdysone levels in Drosophila are not available, but data collected (Fristrom et al, 1974) are consistent with the Calliphora data as far as they go). The high titer of ecdysone at pupariation may be responsible for the expression of DAT at this time, in much the same way as the hormone induces dopa decarboxylase activity. However, the claim has been made that DAT activity is not induced by injection of the hormone into ligated larvae of Calliphora (Karlson & Ammon,1963). In view of our observations that the enzyme is located in the oenocytes, it would be interesting to see if the DAT activity appears in imaginal discs cultured with ecdysone under conditions where dopa decarboxylase is induced (Chen,1974).

No enzyme activities associated with sclerotization can be correlated with the peak of ecdysone found during the pharate adult stage. At this time, adult cuticle is secreted but it is not sclerotized until after eclosion. Hence, tissues which responded to the hormone at pupariation by producing dopa decarboxylase (and possibly DAT) do not so respond at another stage of development.

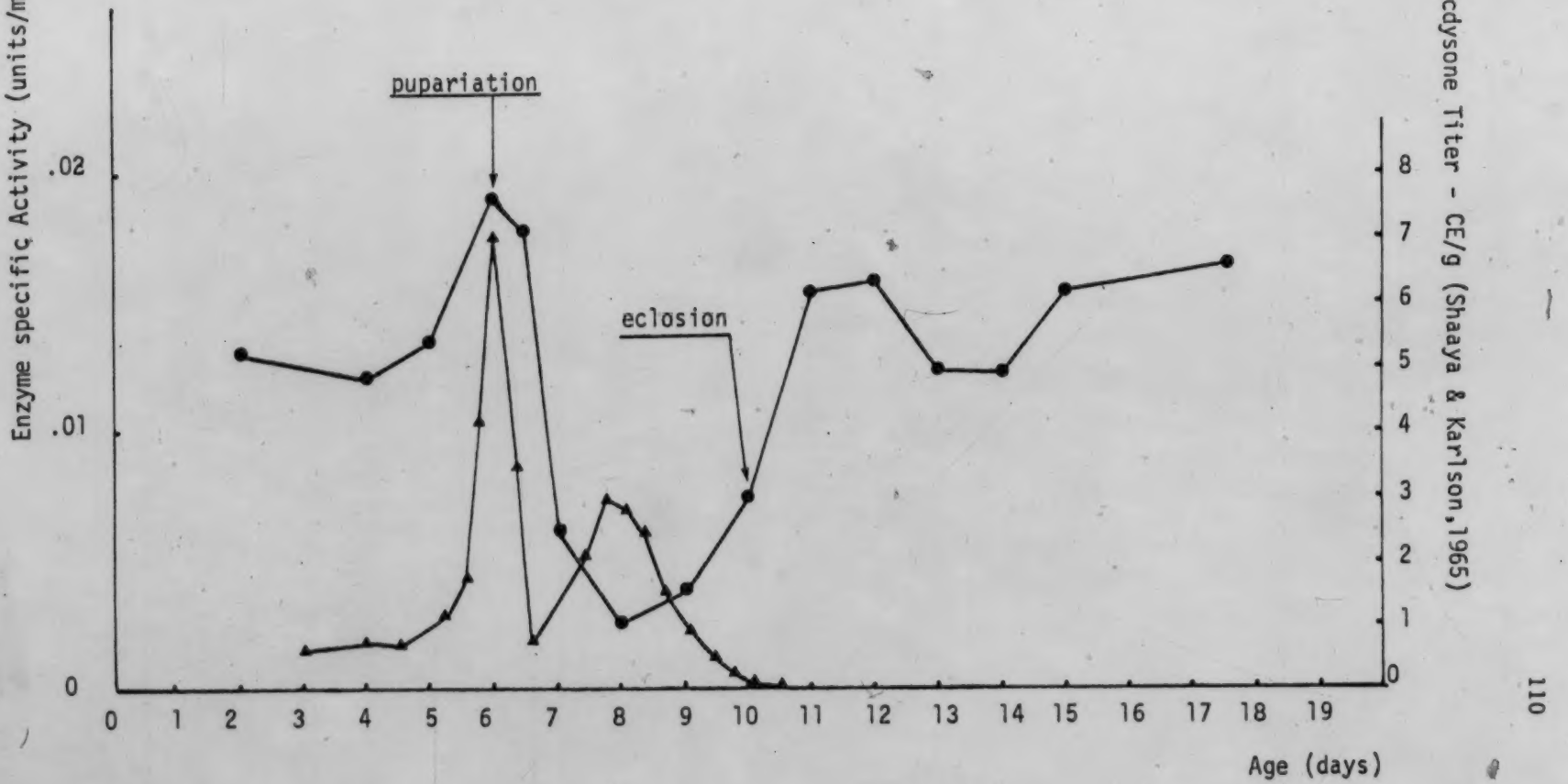
Fig. 29. DAT developmental profile in Drosophila melanogaster compared to ecdysone titer in Calliphora.

The activity profile for DAT was determined at various developmental age in Drosophila melanogaster. Assay conditions were similar to those described in Materials and Methods.

Ecdysone titer was adapted from Shaaya & Karlson (1965) and corrected for differences in time of development for Calliphora ccmpared to Drosophila, pupariation and eclosion being the pcints of comparison.

—o— DAT

—▲— Ecdysone


Enzyme specific Activity (units/min/mg @ 412 nm)
.02
.01
0
pupariation
eclosion
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Age (days)
Ecdysone Titer - CE/g (Shaaya & Karlson, 1965)
8 7 6 5 4 3 2 1 0

The hormone may initiate some events during this pharate adult period, which are preconditions for the expression of the cuticular enzymes at eclosion. A mechanism which readily suggests itself is the translation of a "masked" messenger produced at the earlier time under the action of ecdysone. Alternatively, bursicon could play a role in the appearance of the cuticular enzymes at eclosion. This hormone is known to be an active mediator in the tanning process in various insects (Fraenkel & Hsiao, 1965; Mills & Whitehead, 1970).

A rather surprizing finding of this thesis was the observation that DAT was absent from the epidermal cells, since the enzyme preceeding DAT in the sclerotization pathway, dopa decarboxylase, has been shown to be located mainly in the epidermal cells in Sarcophaga bullata (Chen, 1974), and Drosophila (Lunan & Mitchell, 1969). Moreover, the haemolymph showed no enzyme activity while fat bodies contained only minimal amount of DAT.

Using Sarcophaga bullata , because of their large size, it was possible to dissect out the muscles and the epidermal cells and to demonstrate that most of the DAT activity was present in the muscle fraction while a good proportion of dopa decarboxylase was in the epidermal

cells. Considering the cytological aspect of the cuticle and its immediate surroundings, we hypothetized that the DAT could be located in the oenocytes,large cells sandwiched between the cuticular epidermal cells and the muscle layer. In the dissection process, it is likely that these cells were pulled out with the muscle layer, thus explaining our finding that most of the DAT activity resided in the muscle portion. Oenocytes are large cells and probably fragile, which would account for the ease with which the enzyme was liberated into solution upon grinding. It is surprizing, though, to find such two closely related enzymes , DAT and dopa decarboxylase, in adjacent but distinct and different tissues. Usually, enzymes involved in the same biochemical pathway will be located in the same cells and tissues (Bernhard,1968). However, a definite localization of these enzymes has not been completed yet and to resolve this problem, more elaborate techniques offering higher resolution should be used; for example, immunofluorescence with antibodies specific to DAT or histochemical staining.

Despite the fact that the activity of many genes in Drosophila melanogaster is sensitive to gene dosage (see Hodgetts,1975,for references), we were unable to demonstrate that DAT activity responded to an increase in

gene dosage. It is possible that the locus lies in the triplo-lethal region, 82C-83EF. Alternatively, more than one region of the genome could be responsible for the enzyme activity. For example, if an activator is required in stoichiometric amounts for the expression of DAT, the enzyme activity will not respond to gene dosage if the locus for the activator is not near the structural gene. Moreover, as pointed out by O'Brien and Gethmann (1973), the gene investigated has to be nuclear since for a mitochondrial enzyme, the rate limiting step in enzyme activity might not be gene dosage but the number of mitochondrial sites of enzyme action. Finally, oenocytes contain polytenized chromozomes and the degree of polyteny for the various chromosomes fragments in the aneuploid may not be the same, thus confusing the gene dosage relationship between aneuploid and euploids.

These genetic results were disappointing in that the development of DAT as a new gene-enzyme system in *Drosophila*, a major goal of this thesis, was not forthcoming. However, we feel that *in vitro* development of a reliable and relatively simple assay for this enzyme will allow others to pursue these studies and perhaps further clarify the role of DAT in the sclerotization process.

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